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INNO-SAFE-LIFE

AN INTERDISCIPLINARY AND TRANSLATIONAL EUROPEAN FRAMEWORK FOR POLLUTION-FREE SOILS GENERATING SAFE RESOURCES FOR FOOD SECURITY AND HUMAN HEALTH SUPPORT

Partnership for innovation on the exchange of best practices and the design of joint collaborative initiatives at European level related to the awareness of the effects of contamination on human health.

Erasmus+ Project – Partnership for Coperation

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INNO SAFE LIFE

An interdisciplinary and translational European framework for pollution-free soils that generate secure resources for food security and support of human health

Project title: Partnership for innovation on the exchange of best practices and the design of joint collaborative initiatives at European level related to the awareness of the effects of contamination on human health

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This interdisciplinary course explores innovative European approaches to achieving pollution-free soils that sustain food security and protect human health. It integrates environmental science, ecology, and public health through four key themes: (i) the impact of soil contaminants and cutting-edge ecological strategies for their remediation; (ii) the challenge of antimicrobial resistance driven by both natural and human factors, with a focus on ecological solutions; (iii) the toxicological effects of environmental contaminants on the human body and green methods for their mitigation; and (iv) the importance of healthy nutrition and the use of safe, scientifically approved dietary supplements to promote overall well-being.

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CONTENT

CHAPTER 1. THE IMPACT OF SOIL CONTAMINANTS AND INNOVATIVE GREEN METHODS FOR CONTAMINANT REDUCTION

1.1 Introduction and Key Concepts

Plants can be impacted by pathogens at various stages of their growth, influenced by factors such as weather conditions that may increase the presence of pathogens in the environment, as well as the sanitary practices employed in crop management. For instance, inadequate integration of residues from previous crops can contribute to this issue. A plant's vulnerability to infection arises when environmental conditions disrupt its physiological processes, leading to impaired growth and altered functions (Nazarov et al., 2020). Additionally, the occurrence of plant pathogens is often enhanced by favorable conditions such as optimal temperature, soil pH, and moisture levels. It is important to note that the ideal conditions for pathogen outbreaks can vary significantly between different pathogens.

Soil-borne pathogens are infectious agents that infect host plants via the soil, as opposed to through water or air. These pathogens cause diseases that can significantly reduce agricultural yields worldwide (Li et al., 2023). They typically enter their hosts through the roots, with some pathogens being specific to certain host plants while others can infect a wider variety of species. The mechanisms of infection include the use of molecular signals to assess the susceptibility of plant roots, entry through damaged areas (which may be caused by animals or machinery), or, in some cases, the production of enzymes that break down the chemical compounds in the plant root cell walls. Successful invasion occurs when the pathogen effectively inoculates and disseminates throughout the plant.

The predominant soil-borne pathogens impacting plants are fungi; however, plant diseases can also arise from bacteria, protozoa, viruses, and nematodes. Additionally, noninfectious diseases may occur in the soil due to unfavorable growth conditions and are generally not transmitted from diseased plants to healthy ones. In contrast, infectious soil diseases can be transmitted from a diseased plant to a healthy one

(Nazarov et al., 2020). It is important to note that alterations in soil factors can trigger outbreaks of both infectious and noninfectious soil pathogens.

Soil-borne pathogens can cause a variety of symptoms in plants, which may vary depending on the specific pathogen and the type of plant affected. Common symptoms include: pre-emergence damping-off, post-emergence damping-off, root rot and vascular wilts.

Pre-emergence Damping-off:

Pre-emergence damping-off is a plant disease that primarily affects seeds and seedlings before they emerge from the soil. It is caused by various soil-borne pathogens, including fungi such as Pythium, Rhizoctonia, and Fusarium.

Damping-off is a disease that causes the decay of germinating seeds and young seedlings. It can cause a significant yield losses for farmers, affecting both nurseries and field crops.

Pre-emergence damping-off is more prevalent in conditions that promote high moisture levels, such as overwatering, heavy rainfall, or compacted soils. Poor soil drainage and high soil temperatures can also exacerbate the problem (Punja et al., 2021, Scott and Punja, 2023.)

Symptoms of pre-emergence damping-off:

- Seed, bulb and tuber decay (Figure 1.1 and 1.2): Seeds, bulbs and tubers may rot in the soil before they have a chance to germinate. This decay is often characterized by a mushy texture and discoloration.
- Poor Germination (Figure 1.3): Affected seeds may fail to germinate altogether, leading to uneven or sparse plant stands.
- Seedling Collapse (Figure 1.4): If seedlings do emerge, they may exhibit wilting or collapse shortly after breaking through the soil surface. This is often due to root rot or stem lesions caused by the pathogens.
- Discoloration (Figure 1.5): Seedlings that do manage to emerge may show signs of yellowing or browning, particularly at the base of the stem or on the leaves.

- Stunted Growth: Surviving seedlings may exhibit stunted growth and overall poor vigor, making them more susceptible to other diseases and environmental stresses.

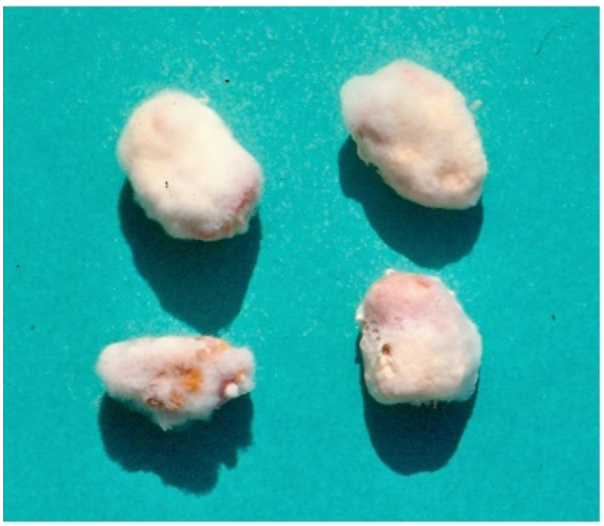

Figure 1.1. Maize seeds decay

Figure 1.2. Narcissus bulbs decay

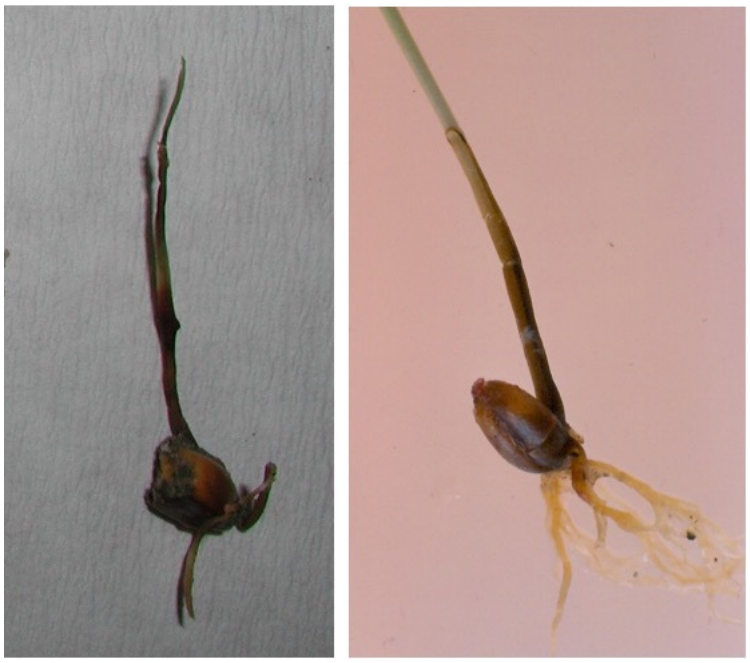

Figure 1.3. Poor germination of maize and wheat seed

Figure 1.4. Seedling collapse

Figure 1.5. Discoloration of seedlings

Post-emergence damping-off

Post-emergence damping-off is a condition that affects seedlings after they have emerged from the soil (Lamichhane at al., 2017). Symptoms typically include:

- Wilting: Seedlings may appear droopy or wilted, even when the soil is adequately moist.

- Stunted Growth: Affected plants often show reduced growth rates compared to healthy seedlings.

- Discoloration: Leaves may turn yellow or exhibit other color changes.

- Soft, Rotted Stems: The stems may become soft and mushy, leading to collapse.

- Root Rot: Roots may appear dark and decayed.

Root rot

Root rot (Figures 1.6, 1.7 and 1.8) is a common plant disease caused by various pathogens that thrive in overly wet conditions.

- Wilting

- Yellowing Leaves

- Stunted Growth: Plants may exhibit slowed or halted growth.

- Dark, Softy Roots: Healthy roots are typically white and firm; affected roots may appear dark, soft, and mushy.

- Foul Odor: A rotten smell may emanate from the soil or roots.

- Overall Decline: The plant may show signs of overall decline, including poor vigor and health.

Figure 1.6. Sugar beet root rot

Figure 1.7. Sclerotinia root rot on sunflower

Figure 1.8. Chalara elegans on rose

Vascular wilts

Vascular wilts are a type of plant disease characterized by the wilting of leaves and stems due to the disruption of water transport within the plant's vascular system.

Common examples of vascular wilt diseases include *Fusarium* wilt (Figures 1.9, 1.10 and 1.11) and *Verticillium* wilt (Figure 1.12). These pathogens can lead to significant agricultural losses as they affect a wide range of crops and ornamental plants.

Figure 1.9. Fusarium wilt of tomato

Figure 1.10. Fusarium wilt of chrysanthemum

Figure 1.11. Fusarium wilt of watermelon

Figure 1.12. Verticillium wilt of sunflower

Disease management

Various measures can be used against soil borne pathogens. They include; agrotechnical measures, mechanical, chemical, physical and biological methods. Integrated Pest Management (IPM): Combining biological control with other management practices and can provide a more effective and sustainable approach to managing soil-borne diseases.

Agrotechnical measures of protection against soil-borne pathogens are essential practices aimed at preventing the spread and impact of these harmful organisms on crops. These measures include:

1. Crop Rotation: Alternating different crops in a specific sequence can disrupt the life cycles of soil-borne pathogens, reducing their populations and preventing disease buildup. In the crop rotation, it is important to avoid producing plant species that are hosts of the same, economically important disease agents. In this case, an increasing occurrence of the disease can be expected from year to year (Figures 1.13 and 1.14).

Figure 1.13. Repeated sowing of wheat results in a high level of disease (*Gaeumannomyces graminis*) occurrence

Figure 1.14. The amount of *Sclerotinia sclerotiorum* inoculum increases by alternating sunflower, soybean and oilseed rape in the crop rotation

2. Soil Management: Practices such as maintaining proper soil moisture, pH levels, and organic matter content can create an unfavorable environment for pathogens. This includes practices like mulching (Figure 1.15) and cover cropping.

Figure 1.15. Mulching

3. Sanitation: Keeping fields and equipment clean helps prevent the introduction and spread of pathogens. This includes removing infected plant debris (Figure 1.16) and disinfecting tools.

Figure 1.16. Removal plant debris from greenhouse

4. Resistant Varieties (Figure 1.17): Planting crop varieties that are resistant or tolerant to specific soil-borne pathogens can significantly reduce disease incidence.

Figure 1.17. Different susceptibility of wheat cultivars to Fusarium head blight

5. Soil Health Improvement: Enhancing soil health through the addition of organic matter, compost, and beneficial microorganisms can promote a balanced soil ecosystem that suppresses pathogens (Figure 1.18).

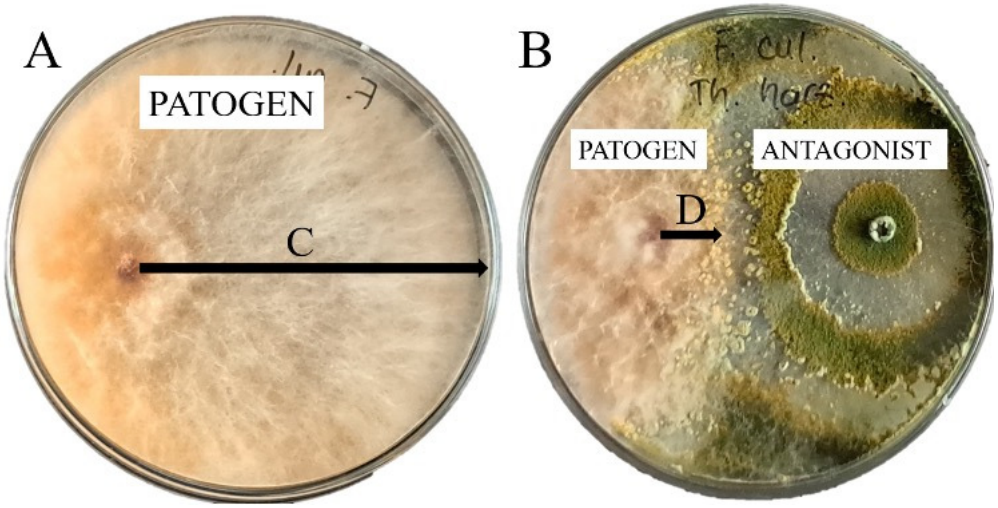

Figure 1.18. Influence of *Trichoderma asperellum* on pathogenic fungus

6. Proper Irrigation Practices (Figure 1.19): Avoiding over-irrigation and ensuring good drainage can help prevent conditions that favor the development of soil-borne diseases.

Figure 1.19. Tobacco irrigation

Mechanical measures some common methods include:

1. Cutting off, uprooting, removing diseased plants from fields (Figure 1.20), plantations, orchards, etc. or plant parts so that they do not become a source of infection for healthy plants.

Figure 1.20. Removing diseased plant parts

2. Destruction of plant organs in which the parasite overwinters, for example from orchards remove all infected leaves after the end of the growing season and bury or burn (Figure 1.21) them.

Figure 1.21. Disposal of diseased plants

Chemical formulations can be apply in the soil as fumigants or as foliar sprays to mitigate the occurrence, spread, and development of diseases in both plants and soil. While these formulations are efficient for preventing significant losses and the outbreak of diseases in crops, they also present several drawbacks. These include ecotoxicity, bioaccumulation, negative impacts on non-target plants and animals, as well as potential risks to human health. (Del Martínez-Diz et al., 2021). The application of chemical pesticides has contributed to the increase in resistant pathogens, which in turn has diminished the effectiveness of many chemical control methods.

The soil can also be treated in an environmentally friendly way such as heated steam, solarization and biofumigation.

Soil-heated steam treatment is an effective method for managing plant pathogens in agricultural settings. This technique involves applying steam to the soil, which raises the temperature sufficiently to kill harmful microorganisms, including bacteria, fungi, and nematodes that can adversely affect plant health (Luvisi et al., 2015). By using this method, the incidence of soil-borne diseases can be reduced, improve soil health, and

enhance crop yields. It is a sustainable alternative to chemical treatments, as it minimizes the risk of chemical residues and promotes a healthier ecosystem.

Soil steaming involves the transfer of energy from combusted fuel through water vapor to elevate the temperature of the soil or substrate to levels suitable for pasteurization or sterilization. At low pressure, steam temperatures exceed 100°C (212°F), and when steam condenses back into water, it releases a significant amount of energy, effectively heating the soil while maintaining minimal moisture content. Pasteurization of soil occurs at temperatures ranging from 71°C to 83°C, while sterilization is achieved at the boiling point of water (100°C). In agricultural practices, soil steaming is primarily regarded as a pasteurization method, with temperature guidelines suggesting 71°C for 30 minutes to eliminate most pathogenic fungi, bacteria, insects, and nematodes, and 83°C for 30 minutes to eradicate resistant weed seeds. However, sufficient time and energy are required to attain these temperatures at the specified soil depth. (Ramón, 2020)

Steam sterilization effectively eliminated many soil organisms; however, certain groups of organisms were able to survive and reproduce. Additionally, this method resulted in an increase in phosphorus levels and soil pH, while potassium levels decreased after the initial sterilization treatment. Despite these changes, steam sterilization remains a rapid and cost-effective alternative to other sterilization methods, particularly when large quantities of soil substrate are required (Dietrich et al., 2020)

Soil solarization is an ecofriendly agricultural practice used to manage soil-borne plant pathogens (Elmore and Katan, 1998). This technique involves covering moist soil with clear plastic for several weeks during warm weather (Figure 1.22), which raises the soil temperature to levels that can kill or suppress various pathogens, pests, and weed seeds.

Research has shown that soil solarization can significantly reduce populations of pathogens such as Fusarium (Bottomley at al., 2024), Pythium, and Rhizoctonia, among others. The effectiveness of this method is influenced by factors such as soil moisture, temperature, and the duration of solarization. During certain times of the year (especially in summer), the soil is covered with transparent polyethylene films to

heat the surface layers of the soil to high temperatures. For maximum soil heating, the film must be close to the ground, which means the soil must be free of debris, plant residues, and large clumps of soil that could lift the film off the ground and potentially damage it. For successful solarization, the soil needs to be well-prepared, loosened to a depth of 30-40 cm, and watered with a larger amount of water. Moist soil conducts temperature better, making solarization more effective.

Figure 1.22. Soil solarization

Biofumigation is an environmentally friendly agricultural practice that involves the use of certain plants, particularly those in the Brassicaceae family including cabbage, cauliflower, broccoli, kale and various mustards), to suppress soil-borne pathogens and pests (Ziedan 2022). This method leverages the natural compounds released by these plants, such as glucosinolates, which can be converted into bioactive substances that have antimicrobial properties when the plant material is incorporated into the soil.

The process typically involves growing specific biofumigant crops, such as mustard or radish, and then incorporating them into the soil while they are still green or shortly after flowering. This incorporation releases volatile compounds that can inhibit the growth of various soil pathogens, including fungi, bacteria, and nematodes, thereby improving soil health and crop yields.

Alternative biofumigation material showed that animal manure, which can produce ammonia, can also be used as a biofumigat material to prevent soil-borne diseases (Zhang at al., 2020).

Biological control represents an effective strategy for managing soil-borne pathogens. At its core, biological control involves utilizing living organisms to target specific plant diseases or pests. The primary mechanisms through which biocontrol agents (BCAs) operate to mitigate soil-borne pathogens include antibiosis, induced systemic resistance, parasitism (specifically mycoparasitism), antagonism, competition for nutrients and space, and the promotion of plant growth through indirect means. Most commercial biofungicides are based on multiple mechanisms of action (Figure 1.23).

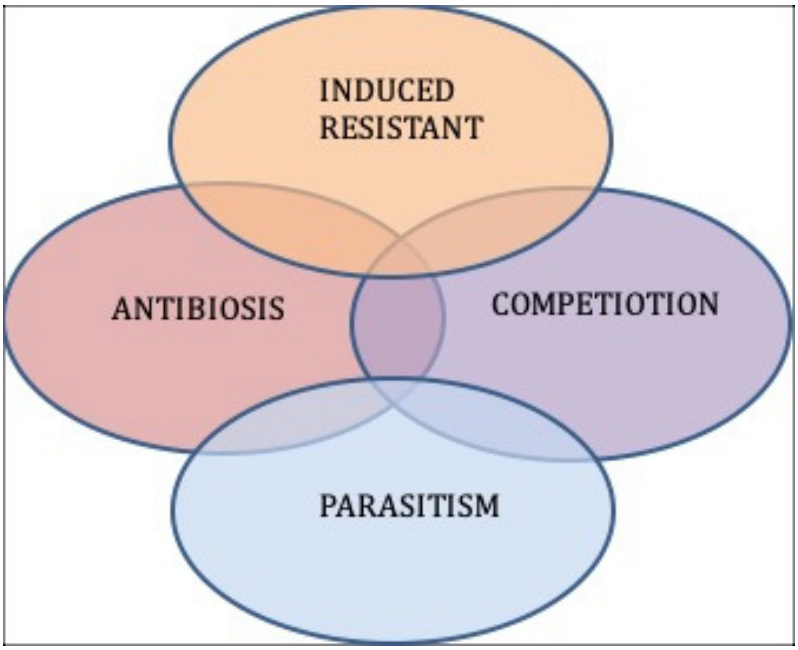

Figure 1.23. Mechanisms of BCA action

The effectiveness of BCAs in field trials is significantly influenced by various ecological factors, including the physiological and genetic state of the host, as well as climatological conditions. These variables contribute to the variability in the impact of BCAs, which is why their application is predominantly confined to greenhouse environments. In greenhouses, environmental conditions can be closely monitored and controlled, allowing for more consistent outcomes (Del Martínez-Diz.et al., 2021)

Biological control is dependent on the various agonistic and antagonistic interconnections between plants and microbes living in the soil. Beneficial soil microorganisms can suppress plant pathogens through two primary mechanisms: direct and indirect actions. Directly, they synthesize metabolites that are antagonistic to the pathogens, such as antibiotics, organic compounds, and toxins. Indirectly, they enhance the host plant's defense mechanisms and inhibit pathogen growth, thereby contributing to overall plant health and resistant.

In commercial biofungicides, of which there are more than 100 worldwide today, the most commonly used antagonistic fungi belong to the genera *Trichoderma, Ampelomyces, Gliocladium, Coniothyrium*, and *Pythium*, as well as antagonistic bacteria from the genera *Streptomyces, Pseudomonas, Agrobacterium,* and *Bacillus.*

The European biopesticides market value increased by about 31.2% during the historical period (2017-2022). The market value is anticipated to increase by about 77.9% and reach USD 3.20 billion by the end of the 2029 year. (https://www.mordorintelligence.com/industry-reports/european-biopesticides-market-industry)

One of the most important and most used genera of fungi in the biological control of pathogens is the *Trichoderma* genus (Figure 1.24). *Trichoderma* employs direct antagonism and competition, especially within the rhizosphere, to influence the composition and interactions of surrounding microorganisms. When colonizing plants, whether on the roots or as an endophyte, *Trichoderma* has developed the ability to communicate with the host plant, providing a range of complex benefits (Woo et al., 2023).

Today there are around 20 *Trichoderma* species in the biocontrol market. Among the registered species, there are *Trichoderma harzianum*, *Trichoderma viride*, and *Trichoderma asperellum* (Sidhu, CABI).

Figure 1.24. Comparison of chemical (left) and biological (right) protection of tomato seedlings against *Rhizoctonia solani* and *Pythium debaryanum*

Trichoderma species are adept at establishing themselves on the rhizoplane, in the rhizosphere, and within plant roots. They generate various metabolites that possess antimicrobial properties, such as enzymes that break down cell walls, antibiotics, and both volatile and non-volatile compounds. They produce biostimulating substances like phytohormones and phytoregulators. *Trichoderma,* in particular, is recognized for its strong capacity to absorb nutrients from roots and engage with not only harmful microorganisms but also the broader soil microbiome.

Bioactive natural compounds can also be used when planting as these promote plant growth and hereby control plant diseases (Pandit et al., 2022). These compounds usually belong to one of three large chemical classes: terpenoids, phenolics, and alkaloids. Some of the plant species used for the extraction of bioactive compounds are; *Allium* spp., *Citrus* spp., *Malaleuca alternifolia* L., brown algae (*Ascophyllum nodosum*), brown algae (*Laminaria digitata*), marine algae (*Spatoglossum variabile*, *Melanothamnus afaqhusainii*, *Halimeda tuna*), red algae (*Chondrus crispus*, *Gigartina stellata*), seaweeds, marine crustaceans (*Crustacea*) (Jamiołkowska, 2020.).

Procedure for registration of plant protection products and risk management

Plant protection products are placed on the market based on the Decision on registration or approval issued by the Ministry of Agriculture of the Republic of Croatia. By registering a plant protection product, the aim is to reduce the risk to human, animal and environmental health, and to encourage integrated and alternative measures to control harmful organisms.

The procedure for registering plant protection products in the Republic of Croatia is the same as the procedure carried out in other European Union members, in order to reduce administrative tasks, a zonal registration system was introduced. In doing so, unique decision-making principles and numerous EU guidelines and standards of international organizations are applied. Documentation of plant protection products must be made according to appropriate guidelines, standards and protocols (GLP, GEP, OECD, FAO, EPPO, and others). Risk assessment and evaluated documentation is the professional-scientific basis for deciding on individual registration or approval of plant protection products.

Regulation (EC) no. 1107/2009 on placing plant protection products on the market was published on November 24, 2009, and its application began on June 14, 2011, with many transition periods. The regulation establishes new standards with the aim of raising the level of safety for the health of people, animals and the environment.

All plant protection products registered in Croatia can be found in the search engine for registered products on the website (FIS database) of the Ministry of Agriculture.

In the Republic of Croatia, three institutions participate in the process of registration of plant protection products (Figure 1.25):

- Ministry of Agriculture;

- Plant Protection Center (Croatian Agency for Agriculture and Food HAPIH);

- Institute for Medical Research and Occupational Medicine (IMI).

The Ministry of Agriculture is the main coordinator of all work related to the registration of plant protection products. The Ministry receives applications for registration, checks the completeness of the documentation and, if necessary, requests additional

documentation from the applicant. Requests and accompanying documentation are submitted in three identical copies. After the completeness of the submitted documentation is determined, two identical copies of the documentation are sent to the authorized institutions for the evaluation of the documentation and risk assessment. In case of need, the authorized institutions request the delivery of additional documentation, certain amendments to requests, explanations, etc. Communication with the applicant in all stages of the plant protection product registration process takes place through the coordinator at the Ministry of Agriculture. After the authorized institutions complete the documentation evaluation and risk assessment, the coordinator submits a proposal for registration to the Ministry of Agriculture. Based on the proposal of the authorized institution, the Ministry of Agriculture issues a decision on the registration of plant protection products.

The Center for Plant Protection performs part of the risk assessment and documentation evaluation. At the request of the Ministry of Agriculture, he prepares proposals for the registration of plant protection products, proposals for issuing certain licenses for plant protection products, proposals for the extension of registrations, and gives expert opinions in the field of plant protection products.

The Institute for Medical Research and Occupational Medicine (IMI) performs documentation assessment and risk assessment in the areas of mammalian toxicology and exposure of users, workers and other persons. At the request of the Ministry, it also issues proposals for the registration of plant protection products, proposals for the issuance of certain permits, proposals for extending registrations, and gives expert toxicological opinions in its field of work. Evaluators in authorized institutions (ZZB and IMI) (coordinators)

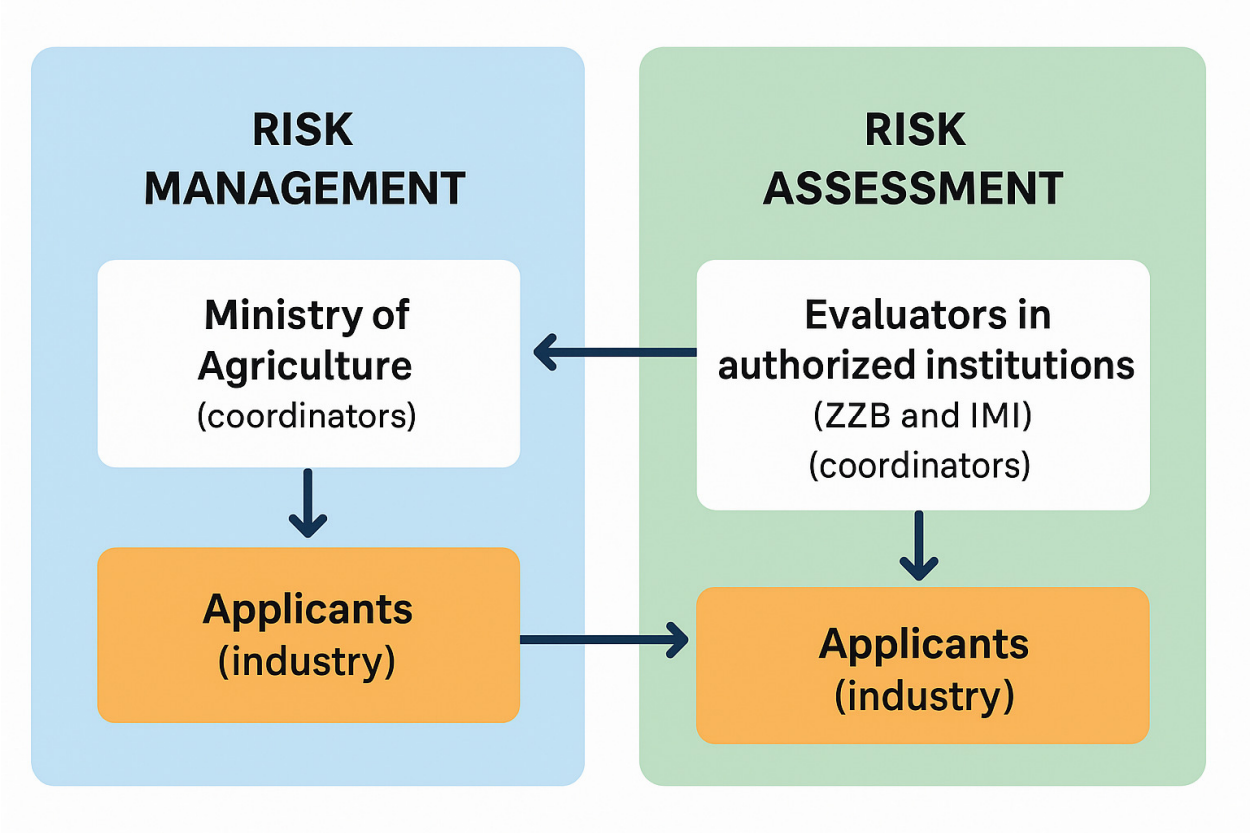

Figure 1.25 Schematic representation of the procedure for registering plant protection products in the Republic of Croatia

Regulation (EC) no. 1107/2009 brings numerous innovations, the most important of which relate to additional criteria for the evaluation of active substances, zonal registration of plant protection products and parallel trade.

The placing on the market of plant protection products in the Republic of Croatia is regulated by the Law on the Implementation of Regulation (EC) no. 1107/2009. This Law has been in force since July 1, 2013 and enables the direct implementation of Regulation (EC) no. 1107/2009 in the Republic of Croatia.

On the basis of Regulation (EC) no. 1107/2009, the European Commission adopted numerous implementing regulations that are directly applicable in the member states of the European Union and which regulate in more detail the placing on the market of plant protection products, approval of active substances, safeners , synergists, coformulants, excipients, lower risk substances, basic substances, zonal registration

system, candidates for replacement, hormonal disruptors (endocrine disruptors), comparative assessment risks, parallel trade, extension of registration for small crops and small purposes, placing treated seeds on the market, data protection, etc.

At the level of the European Union, with the adoption of Directive 91/414/EEC in 1991, unique principles were established for the evaluation of active substances and agents, as well as risk assessment for human and animal health and environmental protection. The implementation of this Directive in the European Union began in 1993.

Registration in the Republic of Croatia and the rest of the European Union

The field of plant protection products and pesticide residues in food at the level of the European Union is governed by Regulations. In June 2013, the following laws were passed that enable the direct application of the Regulation:

Law on the Implementation of Regulation (EC) no. 1107/2009 on placing plant protection products on the market,

Law on the Implementation of Regulation (EC) no. 396/2005 on maximum levels of pesticide residues in and on food and animal feed of plant and animal origin.

Regulation (EC) no. 1107/2009 established a zonal registration system for plant protection products.

The member states are divided into three administrative registration zones (Figure 1.26):

- Zone A - Northern zone, which includes Denmark, Sweden, Finland, Lithuania, Latvia and Estonia

- Zone B - Middle zone, which includes the United Kingdom, Ireland, the Netherlands, Germany, Belgium, Luxembourg, Austria, Slovenia, Slovakia, the Czech Republic, Poland,

Hungary, Romania

- Zone C – Southern zone, which includes France, Spain, Greece, Italy, Portugal, Bulgaria, Malta, Cyprus and Croatia

Administrative zones were established to avoid duplication of work.

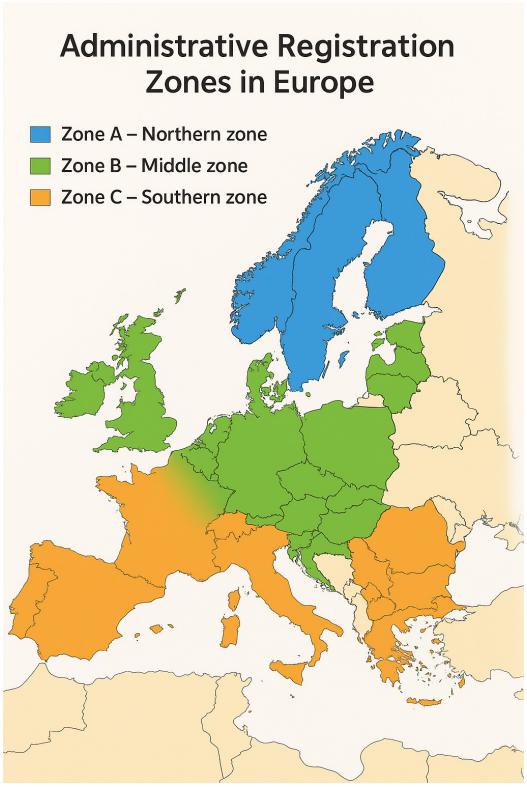

Figure 1.26 Administrative registration zones in Europe

According to Directive 91/414/EEC, each member state of the European Union carried out a documentation evaluation and risk assessment in order to approve the registration of a plant protection product on its territory, and in the zonal registration system, a risk assessment carried out by one member state in the same registration zone or one member state from any registration zone in the interzonal registration procedure. In order to reduce the administrative burden for the industry and for the competent authorities of the Member States and to ensure better uniformity and availability of plant protection products, registrations of plant protection products approved by one Member State should be accepted in other Member States, if they are agricultural, environmental and climatic conditions comparable.

For the above reasons, Member States are divided into zones with comparable agricultural, environmental and climatic conditions, in order to facilitate the mutual recognition of risk assessments and approved registrations. However, the

characteristics of the climate also differ at the level of individual countries, and that is why it is necessary to take these facts into account when registering plant protection products.

Climate is the average state of the atmosphere (gaseous Earth's mantle) over an area observed over a longer period (25 to 35 years). Climate is influenced by numerous factors such as latitude, sea currents, land and sea layout, relief, etc. The Atlantic Ocean is the main source of moisture for Europe. Western winds bring moisture. The amount of precipitation decreases with distance from the Atlantic Ocean. In areas closer to the sea, most precipitation falls in winter, and in more remote areas in summer.

Moderate climates prevail in Europe: moderately warm and moderately continental and Mediterranean climates. Polar and steppe climates are less represented. The climate has an immeasurable influence on the distribution of plant and animal species, the possibility of growing certain plant species in open space, the distribution of pests, plant pathogens and weeds. Plant cover (vegetation) depends on the climate, but human influence is also important. Original vegetation is rare. Man has destroyed large areas of natural vegetation by building settlements and roads and turning forests into pastures and arable land.

An exception to the zonal registration system are those registrations whose uses are not related to climatic and other essential conditions of use. Such uses are seed treatment, use in protected areas, warehouses, silos and the like, and an interzonal registration system has been established for them.

The prerequisite for submitting an application for registration is that the active substance contained in the plant protection product must be approved at the level of the European Union.

The applicant/company applying for registration selects one of the member states within the registration zone as a zonal reporter member state (ZRMS), which performs documentation evaluation and risk assessment for the entire registration zone. Other countries within the registration zone where the Application for Registration was submitted at the same time must refrain from the documentation evaluation and risk

assessment and wait for the zRMS to complete the documentation evaluation and risk assessment. The documentation evaluation and risk assessment process lasts 12 months from the completion of the documentation.

After the zonal reporting state (zRMS) issues a registration to a plant protection product on its territory, other member states in which the company has applied for registration (concerned Member State - cMS) are obliged to issue a registration within 120 days. When deciding on approval conditions, cMS can take into account national specificities and requirements (National Addenda).

In the case when all countries are one zone (use in warehouses and the like), the company chooses one country as an interzonal reporter (interzonal Rapporteur Memeber State - izRMS) for the entire European Union and submits an application to all member states in which it intends to register the protective device herbs. After izRMS issues a registration in its area, each cMS, regardless of the registration zone, is obliged to issue a registration in its area within 120 days.

A member state of the European Union may refuse to issue a decision on the registration of a certain plant protection product for which zRMS or izRMS has made a documentation evaluation and risk assessment. Also, a member state can refuse mutual recognition (MR) of a registration from another member state. In both cases, it is mandatory to inform the applicant and the European Commission of its decision and to submit a professional and scientific explanation of such decision.

The applicant may subsequently submit a request for mutual recognition of the registration in a member state that was not in the capacity of cMS at the time of submitting the request for zonal registration. That member state will recognize the registration and issue a Registration Decision within 120 days or reject the request for mutual recognition.

The regulation also allows the recognition of registrations from another registration zone if the climatic, environmental, agricultural and other conditions of use are similar. However, in order to avoid the "domino effect", recognition of already recognized registrations (recognitions) is not allowed.

For the purpose of evaluating the documentation of plant protection products, for the purpose of their registration or approval in the Republic of Croatia, a risk assessment and evaluation of documentation in the areas of identity, physico-chemical properties, analytical methods, product effectiveness, toxicology, user exposure, product residues, behavior in environment and ecotoxicology. In doing so, unique decision-making principles are applied, as well as numerous EU guidelines and standards of international organizations. The placing of plant protection products on the market of the Republic of Croatia is regulated by Regulations in accordance with the rules and laws of the country.

For the purpose of registration of the plant protection product, part of the submitted documentation should be the Registration Report from the member state (Registration Report), which consists of three parts:

a) National rating of the member state according to which recognition is requested (Part A);

b) Summary of the assessment of the member state according to which recognition is requested (Part B);

c) Confidential data (Part C)

Types of approval according to the Law on the Implementation of Regulation 1107/2009

Temporary approvals - placing on the market products that contain an active substance that has not yet been approved at the EU level in accordance with Art. 30 of Regulation (EC) no. 1107/2009. Complete documentation for the active substance and agent is submitted. The procedure is being abandoned and will only be performed until June 14, 2016.

Zonal approvals when the Republic of Croatia is a zRMS – the Republic of Croatia in the southern administrative registration zone has the role of a reporting country (zonal Rapporteur Member State - zRMS), which performs documentation evaluation and risk assessment for the entire registration zone. The Member States concerned (cMS), in which the application for registration is submitted, are obliged, after commenting and

harmonizing the assessment and after zRMS issues a decision on registration, to approve the device in their countries (member states may have national requirements or may refuse to issue a decision on registration, but they are obliged to inform the European Commission about it).

Interzonal approvals when the Republic of Croatia is from the RMS - all member states represent one zone for the application of funds in protected areas (greenhouses, greenhouses), application after harvest or harvest, application in empty storage areas or areas for storing plants or plant products, and for seed treatment. In the EU, the Republic of Croatia plays the role of an interzonal reporter (interzonal Rapporteur Member State - izRMS), which evaluates documentation and assesses risks for the entire European Union. The concerned Member States (cMS), in which the application for registration is submitted, are obliged, after commenting and harmonizing the assessment and after the izRMS issues a decision on registration, to approve the device in their countries (member states may have national requirements or may refuse to issue a decision on registration, but are obliged to inform the European Commission about it).

Zonal approvals when the Republic of Croatia is cMS – the Republic of Croatia does not evaluate the documentation and risk assessment, but has the right to comment on the evaluation made by zRMS or izRMS. After the completion of the zonal assessment and after zRMS or izRMS issues a decision on registration, cMS has a deadline of 120 days to issue a decision on registration of the asset (the Republic of Croatia can refuse to issue a decision on registration, but it is obliged to inform the European Commission about it).

If the active substance, safener or synergist from the product is from another source or from the same source with a change in the production process and/or place of production, an assessment of equivalence is required, in accordance with Art. 38 of Regulation (EC) no. 1107/2009. Equivalence assessment is carried out at EU-wide level and is usually done by the country that was the RMS for the active substance, but not as a rule. When evaluating equivalence, data on the active substance is evaluated.

Mutual recognition of approval - recognition of approval from another EU member state, according to Articles 40, 41 and 42 of Regulation (EC) no. 1107/2009. The procedure of registration or approval of the means in the country according to which recognition is requested must be carried out according to Regulation 1107/2009 or Directive 91/414/EEC. If the asset has not passed the zonal procedure, i.e. if it is registered in a member state according to Directive 91/414/EEC, the applicant must submit details of the assessment carried out in that country in the form of a detailed national assessment or assessment of part B of the RR or assessment of the MIII document. In cases where there is no national assessment in the member state, according to which the recognition is being made, the documentation assessment and risk assessment are carried out on the basis of the label and license from that country and the submitted MIII document from all areas. It is important to emphasize that in such cases the 120-day period for issuing approval does not apply.

Extension of approval – carried out zonally according to Article 43 of Regulation (EC) no. 1107/2009 for the asset at the request of the owner of the approval, if the conditions from Art. 29 of Regulation (EC) no. 1107/2009, within 3 months after the extension of the approval for the active substance, safener or synergist, which contains that agent.

Withdrawal or amendment of approval - member states may at any time review the approval and withdraw or amend approval according to Article 44 of Regulation (EC) no. 1107/2009. Article 45 of Regulation (EC) no. 1107/2009 allows the withdrawal or amendment of the approval at the request of the owner of the approval, whereby the changes can only be approved if it is still determined that the requirements from Article 29 have been met.

Placing low-risk plant protection products on the market - according to Article 47 of Regulation (EC) no. 1107/2009, when all active substances contained in a product are low-risk active substances according to Article 22, that product is approved as a low-risk product, if no special risk reduction measures are required based on the risk assessment. The requirements for the necessary documentation are smaller according to Article 47, paragraph 1. (a) to (e).

Comparative assessment of plant protection products containing candidates for replacement - is carried out for products containing active substances that have been identified as candidates for replacement. Although the comparative assessment is carried out at the national level, it is not yet carried out in the Republic of Croatia, as guidelines for the Republic of Croatia are currently being developed.

Extension of approval for small purposes - according to Article 51 of Regulation (EC) no. 1107/2009, the owner of the approval, official or scientific bodies engaged in agricultural activity, professional agricultural associations or professional users can request that the approval for the product, already approved in the Republic of Croatia, be extended to small uses that are not yet covered by that approval. The extension can be approved in the form of an amendment to an already existing approval or a separate approval, in accordance with the administrative procedure of the Republic of Croatia. It is possible to extend the authorization for small uses by mutual recognition, provided that the plant protection product concerned is authorized in that Member State. Member States shall authorize such uses in accordance with the provisions of Article 41, provided that such uses are also considered minor in the Member States where the request is made.

Approval for parallel trade - according to Article 52 of Regulation (EC) no. 1107/2009 is issued for the import and placing on the market of a product in the Republic of Croatia, if this product is identical in composition to an already approved reference product in the Republic of Croatia. An assessment of the identity of the means is carried out according to data on the composition of the means and other data obtained from the competent administration from the EU country from which the means are imported into the Republic of Croatia.

Permit in emergency situations - according to Article 53 of Regulation (EC) no. 1107/2009 may be approved, for a maximum period of 120 days, for the placing on the market of plant protection products for limited and controlled use, if such measures prove necessary due to a danger that cannot be contained in any other satisfactory way. It is necessary to inform the European Commission about the above.

License for research and development - according to Article 54 of Regulation (EC) no. 1107/2009 is issued for the purpose of research or development, in which an unapproved agent is released into the environment or an unapproved agent is used, if the member state on whose territory the research or experiments will be conducted has evaluated the available data. Quantities and areas of treatment may be limited, and additional conditions may be prescribed to prevent adverse effects on human and animal health or unacceptable effects on the environment. It is issued only at the national level.

Placing on the market and use of auxiliary means - according to Article 58 of Regulation (EC) no. 1107/2009 is carried out as well as the procedure for approving funds through the zonal procedure.

Assessment of documentation and risk assessment by area

HAPIH – Center for Plant Protection carries out documentation assessment and risk assessment tasks in the following areas:

- the identity of active substances and plant protection agents,

- analytical methods,

- effectiveness of plant protection products,

- pesticide residues in food,

- behavior in the environment,

- ecotoxicology,

- exposure of applicators, workers and other persons present.

- technical coordination, preparation of reports and expert opinions

The European and Mediterranean Plant Protection Organization (EPPO) has divided agroclimatic zones in Europe, the Mediterranean and Eurasia. These zones were created for the international exchange of data on the effectiveness and phytotoxicity of plant protection products. According to the EPPO division, 4 agroclimatic zones have been established:

1. Mediterranean zone,

2. Marine zone,

3. Northeast zone,

4. Southeast zone.

The Mediterranean zone includes countries or parts of countries around the Mediterranean Sea including Jordan, Macedonia and Portugal.

The maritime zone is north of the southwest coast of France, via Lyon to the southern border of Switzerland and Austria, west to the border of Austria and Hungary, further west of the border between the Czech Republic and Slovakia west of the Oder River (the border between Poland and Germany). This zone also includes the entire territory of Ireland, Sweden and the United Kingdom.

The north-eastern zone includes countries and regions east of the Oder River (the border between Poland and Germany), north of the border of the Czech Republic and Poland, west of the border of Poland and Ukraine, north of the border of Ukraine and Belarus, Russia north of 50□ latitude.

The southeastern zone includes Bulgaria, Hungary, Serbia, Moldova, Romania, Russia south of 50□ latitude, Ukraine, Slovakia, Slovenia, Croatia, Bosnia and Herzegovina, Montenegro and Turkey, except for the Mediterranean coastal areas. These areas indicate that they have different climate characteristics than the zone in which the countries are classified. In some member states, e.g. in France and Croatia, these differences are particularly pronounced. Efficiency data from one zone can be considered acceptable in another zone if agricultural, environmental, climatic and other conditions are comparable (Manual for safe handling and application of plant protection products, Ministry of Agriculture 2014).

Assessment of documentation in the area of pesticide residues in food

By applying plant protection agents (pesticides) to treated plants, products of plant and/or animal origin, residues of active substances and/or metabolites remain that can have a harmful effect on human and animal health. Adhering to the prescribed doses

for application, the number and terms of treatment, compliance with the withdrawal period and application only on crops where the plant protection product is approved, it will depend on whether the residues will be in accordance with the maximum permissible amounts of pesticide residues (MDK) or whether they will be in the food found in impermissible concentrations that can potentially endanger the health of consumers, especially sensitive groups such as children, pregnant women, the sick and the elderly.

The use of plant protection products leads to possible exposure to residues directly and indirectly. Residues may remain after use of SZB on/in crops and stored products. Such crops/products are directly used for human consumption or serve as raw material for food and drink. Exposure through drinking water can occur through contact with surface water during application or when pesticides (or their metabolites) are leached into groundwater.

Indirectly, residues in treated crops intended for animal feed can lead to the transfer of residues to/on animal products that are ultimately used for human consumption (such as meat, eggs and milk). Data on the residues of SZB (plant protection products) are needed in order to be able to assess their 'nature' and quantity. Residues can occur when SZB is applied to/in agricultural crops (which are intended in whole or in part for consumption); crops that (can) be fed to livestock (residues in animal products); crops that follow in the same field after treated crops by transfer from the soil (the next crop in the crop rotation); stored foodstuffs or raw materials of food products; drinking water (by drifting pesticides into waterways or leaching SZB into groundwater).

All residue investigations should be supported by validated analytical methods and residue stability studies during storage.

Based on the "residue package" data on residues in food of plant and animal origin and processed products (data on research on metabolism in plants and domestic animals, research on residues by feeding domestic animals (ANIMOD) model, necessary research on residues carried out in accordance with the proposed application, research of pesticide residues in the crop rotation, research during product processing (setting the transfer factor), research of residues of active substances

and/or metabolites in honey) data on the residues of the agent and active substances that are necessary are obtained for assessment in the area of residues.

Assessment documentation in the field of residues is carried out in accordance with the EU Guidelines:

Documentation in the field of pesticide residues in/on treated products, food and animal feed is necessary in order to be able to assess the risk to the health of people, of all age groups, by ingesting product residues in food of plant and/or animal origin.

Climate characteristics and meteorological factors also have an influence on pesticide residues in treated products and soil. The same amount of pesticide applied and the same number of treatments can result in different concentrations of pesticide residues due to agroclimatic differences.

One of the significant parameters that affects the behavior of residues is the climatic difference between production areas. According to Directive SANTE/2019/12752, Europe is divided into two zones for the purpose of evaluating residues after application of the agent in open space:

1. Northern zone – Northern and Central Europe, which includes Sweden, Norway, Iceland, Finland, Denmark, the United Kingdom, Ireland, northern France, Belgium, the Netherlands, Luxembourg, Germany, Poland, the Czech Republic, Slovakia, Austria, Hungary, Switzerland, Estonia , Latvia, Lithuania, Romania and Slovenia and

2. Southern zone – Southern Europe and the Mediterranean, which includes Spain, Portugal, southern France, Italy, Greece, Malta, Croatia, Serbia, Bosnia and Herzegovina, Macedonia, Montenegro, Kosovo, Albania, Turkey, Bulgaria and Cyprus.

The zonal system of registration of plant protection products from the area of residues is divided into two zones

- Northern and Central Europe

- Southern Europe and the Mediterranean

For uses in protected areas (greenhouses, greenhouses) as well as for uses after harvesting, one zone applies. This means that for the purposes of registration of plant

protection products, pesticide residue studies can be done in any zone because the conditions are controlled.

Maximum Residue Levels (MRL)

In order to prove that the requested application of the product is in accordance with the EU regulations on MRL, it is necessary to refer to the assessment of residue studies at the EU level or to submit research studies of residues after the proposed application. The submitted studies should show that the proposed application of the agent will not lead to exceeding the MRL value. The calculation of MRL values is carried out in accordance with the OECD MRL calculator Guidelines with the aim of harmonizing the calculation of maximum residue limit values (MRL) in the Organization for Economic Cooperation and Development, the OECD developed the MRL calculator (https://www.epa.gov/pesticide-tolerances /oecd-maximum-residue-limit-calculator).

MRL values are prescribed by the Law on the Implementation of Regulation (EC) no. 396/2005 on the maximum levels of pesticide residues in and on food and animal feed of plant and animal origin (Official Gazette 80/13), which for the proposed application of the product is harmonized with the European Commission Regulation 396/2005 and accompanying amendments.

According to Regulation (EC) no. 396/2005, maximum residue levels (MRLs) are the upper levels of pesticide residues that are legally allowed in or on food or feed, based on good agricultural practice (GAP) and the lowest exposure necessary to protect vulnerable consumers. They are performed after a comprehensive assessment of the properties of the active substance and the intended use of the pesticide. These legal restrictions also apply to imported food, set as "import tolerances" to meet the needs of international trade.

Before setting or changing MRLs (eg an applicant requests the authorization of a new plant protection product), EFSA assesses the behavior of pesticide residues and the possible health risks to consumers from residues in food. If EFSA's risk assessment does not identify any unacceptable risks to consumers, EU-aligned MRLs are set (EU Pesticide Database MRLs) and the plant protection product can be approved.

Assessment of chronic and acute dietary exposure

The Republic of Croatia does not have its own national nutrition model, risk assessment during product registration is carried out using EFSA's "PRIMo" model (rev3.1) (Pesticide residue intake model http://www.efsa.europa.eu/en/mrls/mrlteam.htm).

When assessing the risk to humans, due to exposure to pesticide residues through food consumption, data on the residues of plant protection agents are compared with the toxicological reference values of the Acceptable Daily Intake (ADI) and the Acute Reference Dose (ARfD).). From the point of view of food safety, a certain type of food is considered safe for consumers if the estimated intake of the harmful substance does not exceed the ADI or ARfD value.

Risk assessment for consumers is carried out by EFSA's "PRIMo" model rev3.1. (Eng. Pesticide Residue Intake Model) as part of the evaluation of the documentation, that is, for the purposes of registering the asset, as part of the National Residue Monitoring Program implemented by the Ministry of Health and for the purposes of the initial risk assessment for the RASFF system.

Cumulative risk assessment

There is a growing need to address the potential risks of combined exposure to multiple pesticide residues in the diet. The available evidence suggests that the main problem is the addition of doses of those compounds that work by the same mode of action. Risks to consumers from the presence of pesticide residues in food are currently assessed on a substance-by-substance basis. However, many pesticides have similar effects and their effects on human health may be greater in combination than individually.

Evaluation of documentation in the field of environmental behavior

Regulations (EU) no. 545/2011 and 284/2013, the requirements regarding the submission of the necessary data on plant protection products, in accordance with Regulation (EC) no. 1107/2009. The provided information on the plant protection product, together with other essential information, as well as the information provided for the active substance, must be sufficient to enable the assessment of the behavior of the product in the environment. All relevant information on the plant protection

product and the active substance must be taken into account for the assessment of expected environmental concentrations (PEC).

In accordance with the document Commission communication in the framework of the implementation of Commission Regulation (EU) No 284/2013 of 1 March 2013 (2013/C 95/02), the methodology for conducting research on the behavior of agents and active substances in the environment is carried out, and all the necessary information is available about guidelines.

Regulations (EU) no. 544/2011 and 283/2013, the requirements regarding the delivery of the necessary data on active substances, in accordance with Regulation (EC) no. 1107/2009. The information provided on the active substance must be sufficient to investigate the behavior of the active substance in the environment (eg the manner and rate of degradation in soil, mobility in soil, route and rate of degradation in water systems are assessed).

Assessment of documentation in the field of ecotoxicology

For the purposes of registration of plant protection products in the field of ecotoxicology, it is necessary to submit

- risk assessment for birds, which should be done according to the EFSA guidelines Risk Assessment for Birds and Mammals, EFSA-Q-2009-00223;

- risk assessment for mammals should be done according to the EFSA guidelines Risk Assessment for Birds and Mammals, EFSA-Q-2009-00223.

- risk assessment for aquatic organisms. For applications submitted before January 1, 2015, the risk assessment for aquatic organisms should be made according to the guidelines SANCO/3268/2001 rev 4. (final) 17 October 2002. For applications submitted after January 1, 2015, EFSA prepared the first of the total three new guidelines for aquatic organisms Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters (EFSA Journal 2013;11(7):3290).

- risk assessment for bees according to the currently valid guidelines SANCO/10329/2002 rev 2 final 17 October 2002. In the event that the studies used in

the higher level risk assessment were conducted in field or semi-field conditions, the exposure in the said studies should be in accordance with proposed GAP for the Republic of Croatia.

- risk assessment for non-target arthropods according to the currently valid ESCORT 2 and SANCO/10329/2002 rev 2 final 17 October 2002 guidelines.

The risk assessment is done for the fauna of non-target arthropods that are "in the field" and "outside the field".

In case the risk assessment shows that there is a risk for non-target arthropods "in the field", it is necessary to submit studies that will prove the recovery of the fauna of non-target arthropods "in the field".

In case the risk assessment shows that there is a risk to non-target arthropods "outside the field", risk reduction measures should be applied.

- risk assessment for earthworms and microorganisms in the soil according to the currently valid guidelines SANCO/10329/2002 rev 2 final 17 October 2002.

- risk assessment for non-target plants according to currently valid guidelines SANCO/10329/2002 rev 2 final 17 October 2002.

Assessment of documentation in the field of toxicology

The Institute for Medical Research and Occupational Medicine (IMI) together with the Center for Plant Protection (HAPIH) performs documentation evaluation and risk assessment in the following areas:

- Toxicology of mammals,

- Exposure of applicators, workers and other persons present

- Technical coordination, preparation of reports and expert opinions

The classification and labeling of the agent is carried out based on the results of studies of acute oral, dermal and inhalation toxicity, eye irritation, irritation and skin hypersensitivity, according to the criteria specified in Annex I of Regulation (EC) 1272/2008 or according to the translation tables from Annex VII of the same

Regulation. When labeling, the warning labels that are transferred to the product based on the toxicological properties of the active substance and/or additives in the formulation, which can be found in Annex VI of the Regulation or in the C&L Inventory database on the ECHA website, should also be taken into account. For all active substances and all additives in the formulation, it is necessary to submit Safety Data Sheets in English in accordance with Annex II of Regulation (EC) 1907/2006 (REACH Regulation), and for plant protection agents it is possible submit also the Safety and Technical Data Sheet in the Croatian language and approved by the Croatian Institute of Toxicology, if it has already been prepared.

As of June 1, 2015, plant protection products must be classified and labeled according to Regulation (EC) 1272/2008, which must be indicated on the label proposal when applying for product registration. Although Regulation (EC) 1272/2008 and together with the first two amendments (Adaptation to Technical Progress - ATP) were transferred to the Ordinance on classification, marking, marking and packaging of dangerous chemicals (Official Gazette 64/11, 137/11, 71/12) , with Croatia's accession to the EU, further additions and changes will no longer be transmitted in the form of an Ordinance, because from July 1, 2013, the Ordinance is directly applicable in the Republic Croatia. All changes to the Regulation can be found on the website of the European Chemicals Agency (ECHA).

The evaluation of the documentation of plant protection products from the field of toxicology is based on the evaluation of the toxicological properties of the active substance and co-formulants in accordance with the requirements prescribed by:

Regulation (EC) no. 1107/2009; Regulation (EC) No. 1272/2008 - classification and labeling of chemicals; EFSA conclusions (Peer Review); Safety - technical sheet (STL/MSDS); Permits and labels of the relevant Member State.

Approval of active substances

The European Chemicals Agency (ECHA), based in Finland, is responsible for the safe use of chemicals by everyone involved in the process (environment, consumers, workers, companies, etc.). ECHA enforces EU chemicals legislation, ensures the

competitiveness of the European chemical industry and innovation, while ensuring independence, transparency and scientific soundness in policy decision-making.

Active substances are still approved at the level of the European Union. The industry prepares the necessary studies for the preparation of documentation and selects a reporting country that represents the company before the European Commission and the European Food Safety Authority (EFSA), which performs risk assessment in all relevant areas, and the European Commission and member states through their representatives on the Standing Committee for the Food Chain and Animal Health - Plant Protection Products - Legislation, discuss and make decisions on the approval of active substances by a qualified majority. Active substances are most often approved for a period of up to ten years and are subject to regular re-evaluation, and in the case of new scientific knowledge, they are subject to re-evaluation even before the expiry of the approval period. The prerequisite for submitting an application for registration is that the active substance contained in the plant protection product must be approved at the level of the European Union.

Evaluations of active substances at the EU level in which the Republic of Croatia is the reporting member state (Reference Member State RMS) and in cooperation with the European Food Safety Authority (EFSA) carries out a risk assessment in the process of approval or re-approval of the active substance substance, while the European Commission plays a risk management role in the procedure and makes the final decision on the approval of an individual active substance at the EU level. Risk assessment tasks for the evaluation of the active substance include the evaluation of documentation in the field of effectiveness, residues, ecotoxicological properties, behavior in the environment, analytical methods, physicochemical properties, toxicology and user exposure according to unique principles, where it is necessary to ensure a high level of protection of human and animal health and the environment.

Endocrine disruptors (EDS) and biocides

Endocrine disruptors (hormonal disruptors) are natural and chemical substances that can change the functions of the hormonal system in humans and animals and thereby adversely affect their health.

In 2002, the World Health Organization (WHO) defined an endocrine disruptor as a substance or mixture that changes the function(s) of the endocrine system and consequently causes adverse effects on the health of an intact organism, its offspring or (sub)populations.

The EU has been dealing with endocrine disruptors for years through legislation in areas such as human health (including consumers and workers), animal health, food and feed, and the environment.

EU regulatory agencies, independent scientific committees, the Commission and Member States already deal with endocrine disruptors regulated by sectoral legislation in areas including human health (including consumer and worker health), animal health and the environment. Examples are EU legislation on safety and health at work (where legislation on chemical agents at work includes all chemical agents, including endocrine disruptors), food and feed safety (where toxicological risks, including those arising from endocrine disruptors, subject to a comprehensive risk assessment) and consumer products (including for example cosmetics and toys, REACH), as well as environmental legislation. On the basis of Regulation (EU) no. 528/2012) on biocidal products, active substances that are considered to interfere with the work of the endocrine system will not be approved, unless it is determined that the risk of exposure to the active substance is negligible or if there is evidence that the active substance is necessary for the suppression or control of a serious dangers for human and animal health and for the environment. The European Commission has asked ECHA and the European Food Safety Agency (EFSA) to draw up, with the support of the Joint Research Center (JRC), joint guidelines on the application of hazard-based criteria for the identification of endocrine disruptors in the context of Regulation (EC) no. 1107/2009 on plant protection products and Regulation (EU) no. 528/2012 on biocidal products, which were published in the Official Journal of EFSA.

Biocides

Biocidal products (Figure 1.27) are substances and mixtures, prepared in the form in which they are delivered to the user, which contain one or more active substances, the

purpose of which is to destroy, deter, render harmless, prevent the action of, or control any harmful organism chemically or biologically.

Figure 1.27 Bicides in plant protection

Biocides and plant protection agents are pesticides. Biocides are used in communal hygiene (DDD - disinfection, disinsection, deratization), to maintain personal hygiene, to protect materials (e.g. walls of houses from fungi and other pests, boats from leeches or algae, wood from various pests, etc.) and many other places (eg to destroy harmful fish, birds, etc.).

In our country, this area is regulated by the European Regulation on biocidal products.

Biocidal products can also contain other inactive common additives that ensure efficiency, as well as the desired pH, viscosity, color, smell of the final product. Biocidal products are available on the market for the needs of professional and/or non-professional consumers (Wittmer IK et al.; (2011), Christensen FM et al.; Scher, et al., 2012).

The Ordinance on documentation for the assessment of the active substance in biocidal preparations, documentation for the assessment of biocidal preparations,

procedures for assessing biocidal preparations and their use, and on the types of biocidal preparations with their descriptions and unique principles for the assessment of biocidal preparations prescribes the documentation for the assessment of the active substance in biocidal preparations, documentation for the assessment of biocidal preparations, the procedure for assessing biocidal preparations and their use, and the types of biocidal preparations, their descriptions and unique principles for the assessment of biocidal preparations are determined.

Biocidal product evaluation procedure

The competent evaluation body evaluates the documentation, prepares the Draft Report and evaluation conclusions. The draft Evaluation Report is sent to the applicant within 30 days for the submission of written notes.

The evaluation report and the summary of product properties are sent to the expert evaluation of the Committee for Biocidal Products (OBP). The Committee for Biocidal Products has 180 days to issue an opinion based on an expert assessment and to submit it to the Commission. The Commission makes a decision on the approval of the biocidal product in the Union (Figure 1.28).

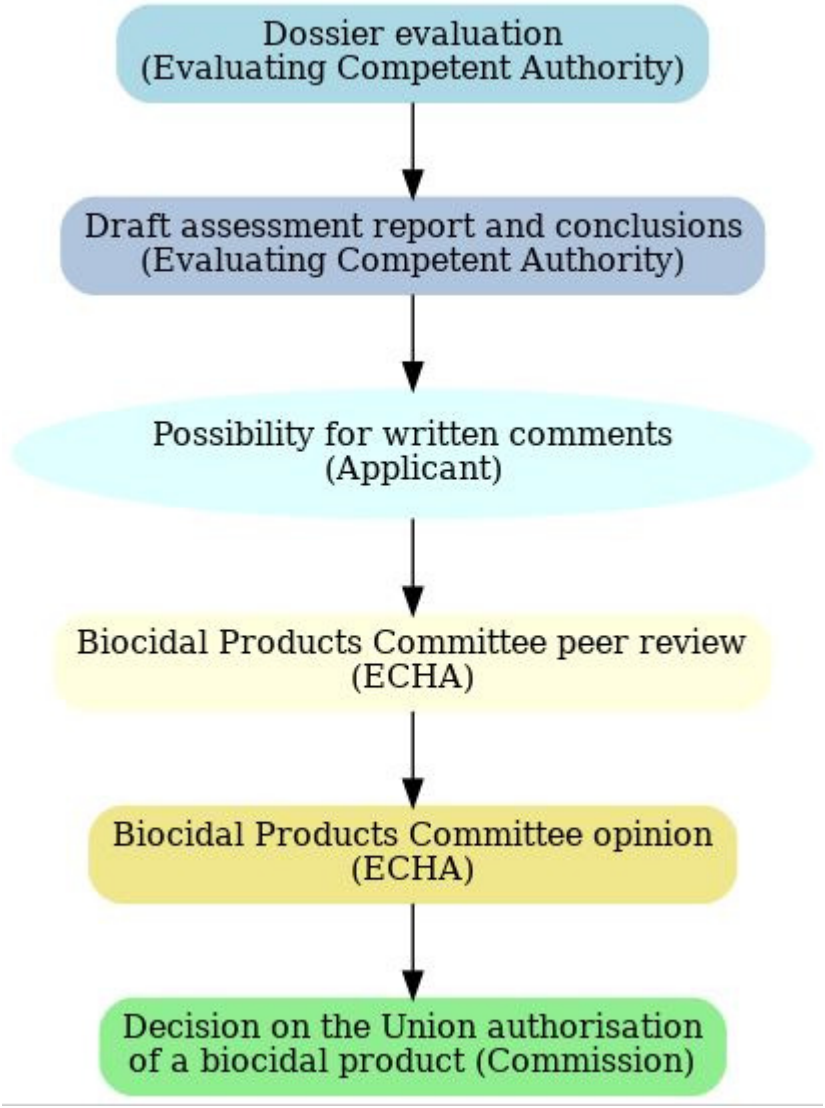

Figure 1.28 Overview of the evaluation process of biocidal product documentation

Requests for the approval of a biocidal product are submitted in the R4BP 3 system (Register for biocidal products), as well as all other requests based on the Regulation on equal biocidal products (BPR).Requirements for entry into the register and placing biocidal preparations on the market, for biocidal products containing active substances listed in paragraph 2 of Article 89 of Regulation (EU) no. 528/2012 are submitted via the web form.

From September 1, 2015, biocidal products cannot be made available on the market, if the manufacturer or importer of the active substance contained in the product or, if

necessary, the importer of the biocidal product is not included in the list maintained by the European Chemicals Agency ECHA Agency-ECHA).

All decisions made in accordance with the Act on Biocidal Products (Official Gazette 63/07, 35/08, 56/10) cease to be valid with regard to the date of approval of a specific active substance for a specific type of product and the disapproval of an active substance in the manner specified in Article 89 of the Regulation (EU) no. 528/2012.

The procedure for registering plant protection products in the Republic of Croatia is the same as that carried out in other European Union members, and in order to reduce administrative work, a zonal registration system was introduced. In doing so, unique decision-making principles and numerous EU guidelines and standards of international organizations are applied. Documentation of plant protection products must be made according to appropriate guidelines, standards and protocols (GLP, GEP, OECD, FAO, EPPO, and others). Risk assessment and evaluated documentation is the professional-scientific basis for deciding on individual registration or approval of plant protection products.

All plant protection products registered in Croatia can be found in the search engine for registered products on the website of the Ministry of Agriculture.

By registering a plant protection product, the risk to human, animal and environmental health is reduced, and integrated and alternative measures to control harmful organisms, plant diseases and weeds are encouraged.

For the purposes of registering a plant protection product in the Republic of Croatia, it is necessary to submit all the necessary studies in accordance with the prescribed Guidelines and Laws, carried out in the southern EU zone, on the basis of which the plant protection product could be approved, because without studies carried out in the southern EU zone, it is not possible to assert whether the means lead to health risks for children and adults.

1.2 Phytoremediation of Heavy Metals in Soils

Phytoremediation is a bioremediation strategy that involves using living plants, algae, or fungi to mitigate environmental contamination, specifically by reducing the concentration of pollutants such as heavy metals, organic compounds, or radionuclides in soil, water, or air (Figure 1.29). This process is driven by the natural capacity of plants and microorganisms to absorb, transform, or sequester these pollutants, facilitating their detoxification and promoting environmental restoration. The use of phytoremediation as a sustainable and cost-effective solution for the remediation of contaminated sites has gained significant attention in recent decades (Vangronsveld, 2009).

The term "phytoremediation" is derived from the Greek word *phytos* (meaning plant) and the Latin word *remedium* (meaning to treat or heal). The process encompasses various mechanisms through which plants, in cooperation with root-associated microorganisms, play a key role in the isolation, transport, detoxification, and mineralization of pollutants within the soil environment (Prasad, 2003).

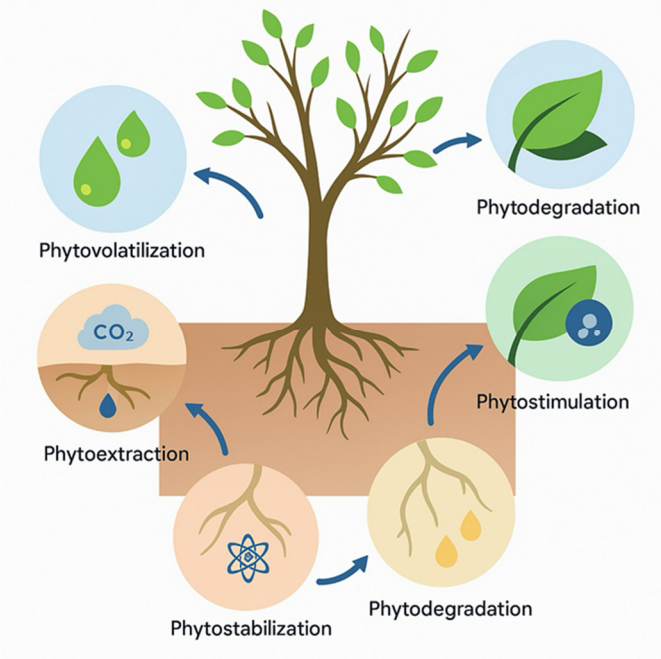

Figure 1.29 Types of phytoremediation

WAYS OF PHYTOREMEDIATION

Remediation of soil contaminated with heavy metals can be carried out by various methods:

PHYSICAL-Soil leaching

CHEMICAL-Bioremediation

BIOLOGICAL-Bioremediation

Examples of these methods (Figure 1.30) include phytoremediation, stabilization, rhizofiltration, phytovolatilization and various soil leaching methods (Vangronsveld, 2009).

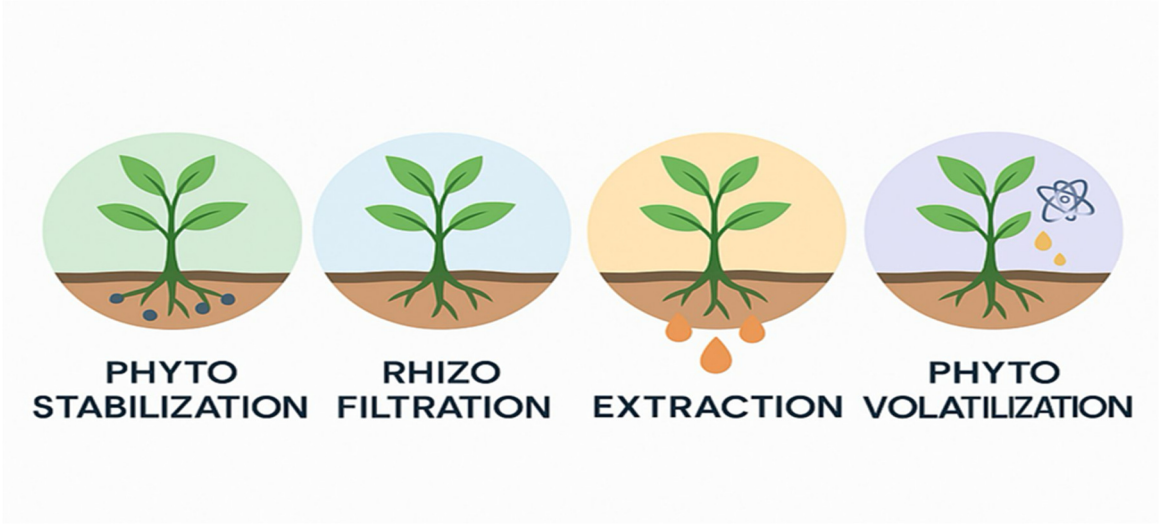

Figure 1.30 Phytoremediation methods

Phytoremediation uses plants, algae and fungi to remove, reduce or transform pollutants (Küpper, 2007).

The basic mechanisms of phytoremediation include:

1. **Phytoproliferation**

Process in which plants grow in contaminated environments, specifically polluted soils or waters, and absorb pollutants through their roots. This process involves the natural ability of plants to tolerate and accumulate pollutants, such as heavy metals, organic compounds, and other toxic substances, from the environment. Unlike mechanisms that involve immobilizing or stabilizing pollutants (like phytorrhizostasis), phytoproliferation focuses on the active uptake of contaminants by plants as they grow. This makes it a key component of phytoremediation strategies aimed at cleaning up polluted sites by utilizing plants' inherent physiological mechanisms to absorb and potentially detoxify pollutants (Cheng, 2002).

Phytoproliferation can be particularly useful in environments where pollution levels are moderate to high, and where other methods of remediation—such as chemical treatments or physical removal—are either too costly or not feasible. Through this process, plants can help improve soil or water quality, making it a sustainable and eco-friendly option for managing contaminated sites .

Mechanisms of Phytoproliferation:

Phytoproliferation is driven by several key mechanisms through which plants absorb and accumulate pollutants from the surrounding environment. These mechanisms are often interrelated and can vary depending on the type of pollutant, plant species, and environmental conditions. The primary mechanisms involved in phytoproliferation include: root uptake and translocation of pollutants, metal and pollutant bioaccumulation, phytoremediation of organic pollutants, enhanced root growth and expansion in polluted soils, rhizosphere interactions and pollutant mobilization (Glick, 2010).

Benefits of Phytoproliferation in Pollution Mitigation:

1. **Pollutant Removal and Contaminant Stabilization:** Phytoproliferation allows for the active removal of pollutants from contaminated sites by concentrating these substances in plant tissues. This is particularly useful for heavy metals, as well as organic contaminants. By absorbing and storing these pollutants,

plants reduce their bioavailability in the environment, thus preventing further contamination of the soil, water, and air. Once pollutants are absorbed and accumulated, the plants may be harvested and disposed of properly, providing a means for long-term management of contaminated sites.

2. **Improved Soil and Water Quality:** Through phytoproliferation, plants help restore the quality of polluted soils and water bodies. In polluted aquatic environments, plants can absorb contaminants directly from the water, improving water quality by reducing pollutant concentrations. Similarly, in contaminated soils, plants can enhance soil structure and reduce erosion, leading to improvements in soil fertility and stability.

3. **Cost-Effective and Sustainable Solution:** Phytoproliferation is an eco-friendly and cost-effective method of environmental remediation. Compared to traditional remediation technologies, such as soil excavation, chemical treatment, or soil washing, phytoproliferation is relatively inexpensive. The use of plants for pollution mitigation also reduces the environmental footprint of remediation processes, making it a sustainable option for long-term environmental management.

4. **Biodiversity and Ecosystem Restoration:** One of the key advantages of phytoproliferation is that it allows for the restoration of biodiversity in polluted environments. By using plants that are native or adapted to the local environment, phytoproliferation can help re-establish natural ecosystems that may have been damaged by pollution. In addition, plant growth in contaminated areas can attract and support wildlife, further contributing to ecosystem restoration and overall environmental health (Figure 1.31)

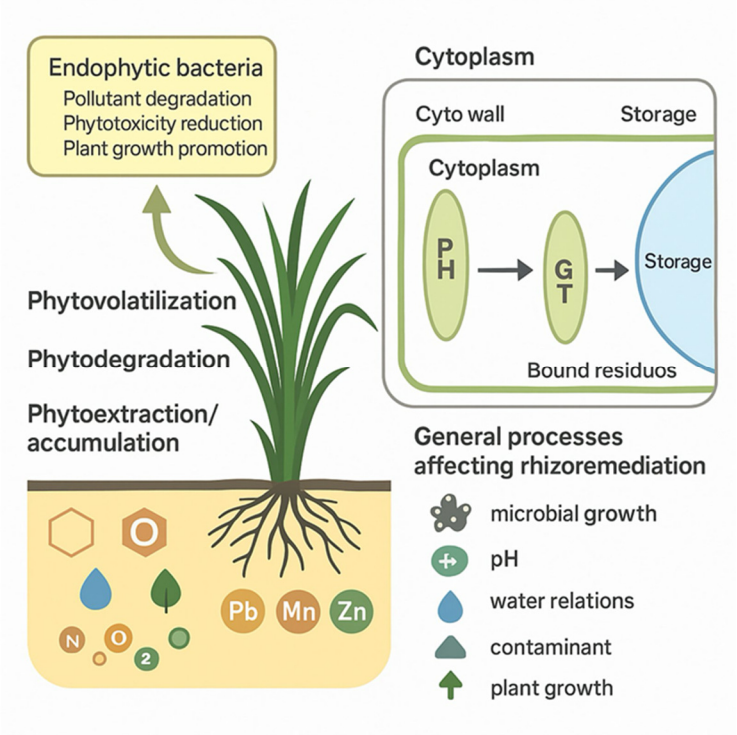

Figure 1.31. Mechanisms of phytoremediation

2. **Phytorrhizostasis**

Phytorrhizostasis is a term used to describe a specific form of phytoremediation where plants accumulate pollutants in their tissues, particularly in their roots, without suffering significant damage. This mechanism serves as an important strategy for immobilizing contaminants, particularly heavy metals, in the rhizosphere and preventing their spread into surrounding areas. By concentrating pollutants in one localized area, plants facilitate the easier removal or containment of these hazardous substances. While phytorrhizostasis is often regarded as a stabilizing technique, it plays a crucial role in the broader field of environmental remediation (Clemens, 2001).

Mechanisms of Phytorrhizostasis:

In phytorrhizostasis, plants do not necessarily "clean" the environment by extracting and removing pollutants from the soil, but instead, they act to contain these contaminants within a specific area. The pollutants are generally retained in the root zone, where they become immobilized, reducing their mobility and preventing them from leaching into groundwater or spreading through soil and air. Several mechanisms

underpin the phytorrhizostatic process such as: root exudation and metal binding, formation of metal complexes, physical containment in roots, alteration of soil properties (Hossain, 2012) .

Benefits of Phytorrhizostasis:

1. **Prevention of Contaminant Spread:** The primary benefit of phytorrhizostasis is its ability to reduce the spread of pollutants. By immobilizing heavy metals or other contaminants in the root zone, plants prevent these substances from being transported through the soil or water, thereby safeguarding surrounding ecosystems. This is particularly important in environments where contaminants are at risk of being carried away by surface runoff or leaching into groundwater.
2. **Minimal Plant Damage:** Unlike other mechanisms of phytoremediation, such as phytoextraction, phytorrhizostasis allows plants to accumulate pollutants without causing significant damage to plant health. The pollutants remain concentrated in the root system, preventing their upward movement into the stems and leaves, where they could impair photosynthesis or growth. This minimizes the negative impact on the plant and ensures that it can continue to function normally in its environment.
3. **Improved Soil Quality:** Phytorrhizostasis can also lead to improved soil quality. By stabilizing pollutants in one area, it reduces the toxicity of the soil in the surrounding areas. Moreover, the root systems of plants involved in phytorrhizostasis can enhance soil structure, increase microbial activity in the rhizosphere, and potentially lead to improved soil fertility.
4. **Cost-Effective Remediation:** Phytorrhizostasis, as part of a broader phytoremediation strategy, is a cost-effective and environmentally friendly approach to managing contaminated sites. This technique requires relatively low maintenance compared to more conventional remediation methods, such as excavation, chemical treatment, or soil washing. Moreover, the plants involved in phytorrhizostasis can be grown on-site, reducing transportation costs and enhancing the overall sustainability of the process.

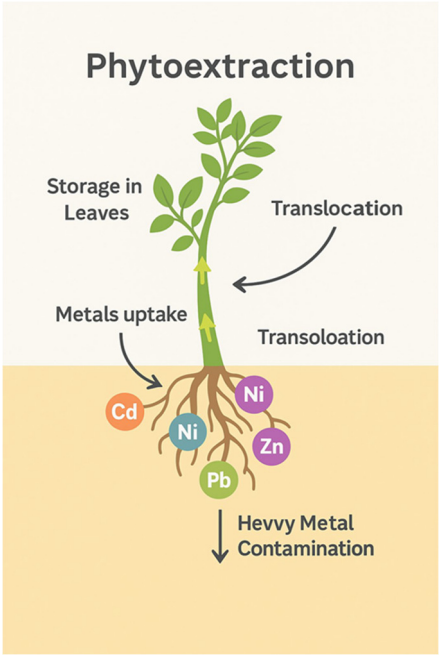

Figure 1.32. Mechanisms of phytoextraction

3. Phytodegradation

Phytodegradations is process in which plants, through the production of specific enzymes, break down or transform pollutants—usually organic contaminants—into less harmful compounds. This mechanism of phytoremediation plays a crucial role in the detoxification of polluted environments by utilizing the natural metabolic pathways of plants to degrade toxic substances. Unlike other phytoremediation mechanisms such as phytoextraction (which involves the accumulation of pollutants in plant tissues), phytodegradation focuses on the biochemical transformation or degradation of pollutants, often leading to the mineralization of contaminants into non-toxic products (McGrath, 2001, McGrath and Zhao, 2003) .

Phytodegradation is an important strategy for remediating sites contaminated with organic pollutants such as pesticides, solvents, petroleum hydrocarbons, and industrial chemicals. Plants capable of phytodegradation are able to metabolize these contaminants directly through enzymatic activity, breaking them down into less toxic or even harmless forms, contributing to environmental restoration (Newman and Reynolds 2004).

Mechanisms of Phytodegradation

Phytodegradation involves several biochemical pathways, whereby plants use their inherent enzymatic systems to degrade or modify contaminants. These processes are facilitated by both plant enzymes and microbial communities in the rhizosphere (the region surrounding plant roots), which can work synergistically with the plant to break down pollutants. Some of the key mechanisms involved in phytodegradation include: enzymatic breakdown of organic pollutants, metabolic pathways for pollutant degradation, role of rhizosphere microorganisms, toxicity reduction and mineralization.

Benefits of Phytodegradation:

1. **Detoxification of Organic Pollutants:** Phytodegradation is particularly useful for the treatment of organic pollutants such as pesticides, herbicides, solvents, and petroleum products. By using plant enzymes to break down or transform these substances, phytodegradation can significantly reduce their toxicity, making the environment safer for wildlife, plants, and humans. The process can also contribute to the breakdown of complex pollutants into simpler, less toxic compounds, effectively removing them from the contaminated site.

2. **Sustainability and Eco-Friendliness:** Phytodegradation is a natural process that requires minimal energy input compared to traditional methods of pollutant removal, such as incineration or chemical treatment. By utilizing plant enzymatic systems, this process is sustainable and eco-friendly. The use of plants and microorganisms for environmental cleanup is a cost-effective and low-maintenance solution that aligns with the principles of green chemistry and sustainable development.

3. **Application to Large-Scale Contamination:** Phytodegradation has the potential to be used in large-scale environmental remediation efforts. Plants can be grown over large areas to absorb and degrade pollutants, making this process suitable for treating contaminated soils, water bodies, and industrial sites. Moreover, certain plants capable of phytodegradation, such as *Populus* species, have fast growth rates and can accumulate large quantities of pollutants, making them ideal candidates for use in remediation programs.

4. **Enhancing Soil and Ecosystem Health:** The degradation of organic contaminants through phytoremediation can improve soil health by reducing the levels of toxic chemicals that inhibit soil microbial activity and plant growth. This process can also help restore ecosystems that have been degraded by pollution, leading to the recovery of biodiversity and ecosystem function (Figure 1.33).

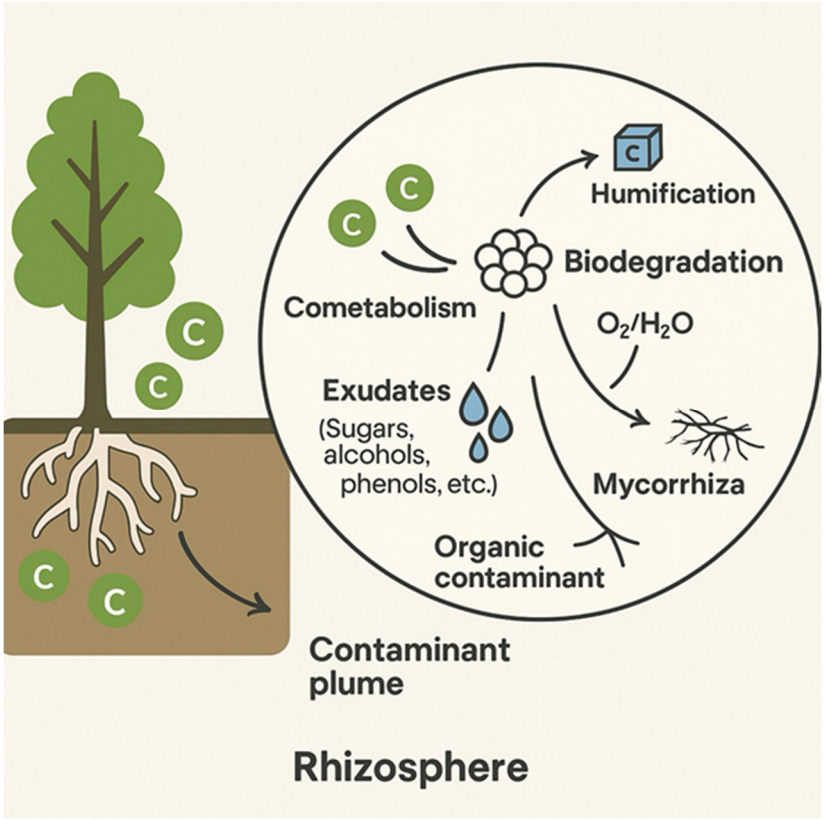

Figure 5. Mechanism of remediation in the rhizosphere

4. **Phytoextraction**

Phytoextraction is process in which plants absorb contaminants, typically heavy metals or toxic elements, from soil or water through their root systems and accumulate them in their above-ground tissues, such as leaves, stems, and flowers. The key aspect of phytoextraction is that after the pollutants are absorbed and concentrated in the plant, the plants are harvested and removed from the contaminated area, thus physically removing the pollutants from the environment. This mechanism is an important part of

phytoremediation, offering a sustainable, eco-friendly method to clean up contaminated sites (Raskin, 1994).

The primary pollutants targeted by phytoextraction are typically heavy metals like lead (Pb), cadmium (Cd), arsenic (As), mercury (Hg), and nickel (Ni), as well as other toxic elements. These metals are often non-degradable and can persist in the environment, causing long-term pollution of soils and water bodies. Through phytoextraction, plants can efficiently remove and concentrate these contaminants in their biomass, which can then be safely disposed of or processed, thus reducing the overall toxicity of the contaminated area.

Mechanisms of Phytoextraction:

Phytoextraction (Figure 1.32 and 1.34) involves a series of steps in which plants interact with pollutants in the environment, absorb them, and concentrate them in their tissues. Several key mechanisms are involved in this process: root uptake of pollutants, transport of pollutants to above-ground tissues, concentration of pollutants in plant tissues, harvesting and disposal of contaminated plant material (Gonzlez, 2008).

Benefits of Phytoextraction:

1. **Cost-Effectiveness and Sustainability:** One of the key benefits of phytoextraction is its cost-effectiveness compared to traditional remediation methods such as excavation, chemical treatments, or soil washing. Phytoextraction requires less energy and infrastructure, making it a more sustainable and economically viable option, especially for large-scale applications. Plants can also be grown on-site, reducing the need for transportation and other logistical challenges.
2. **Non-Disruptive and Environmentally Friendly:** Unlike mechanical or chemical remediation techniques, phytoextraction is non-invasive and minimally disruptive to the environment. It does not involve the removal or destruction of the soil structure, which can lead to soil erosion and habitat destruction. Additionally, the use of plants for remediation contributes to carbon sequestration, which is an added environmental benefit.

3. **Selective Removal of Contaminants:** Phytoextraction can be selective, allowing for the removal of specific pollutants without disturbing other components of the ecosystem. By using hyperaccumulators, it is possible to target specific metals or contaminants for extraction, ensuring that the cleanup process is highly efficient and focused.

4. **Restoration of Soil Health:** Through phytoextraction, plants can help restore the health of contaminated soils. As pollutants are removed from the soil, the soil's physical and chemical properties can improve, making it more suitable for future plant growth and reducing the risk of toxic exposure to surrounding ecosystems.

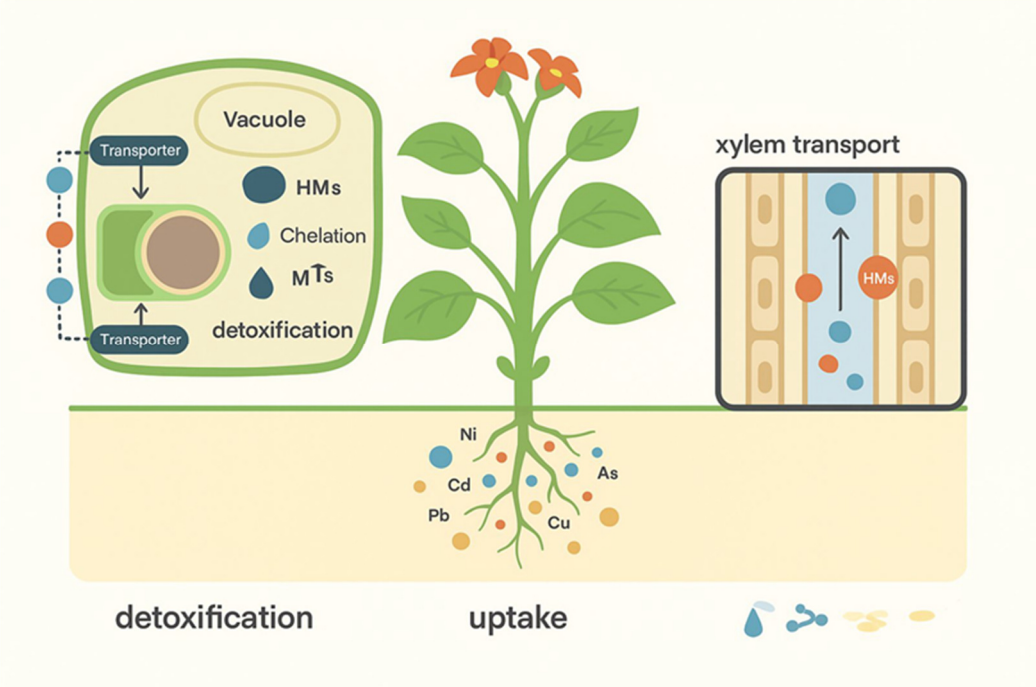

Figure 1.34 Remediation mechanism within plant tissue

5. **Phytorizofiltration**

Innovative bioremediation technology that leverages the natural ability of plants to absorb, accumulate, and detoxify pollutants from water through their root systems. This process not only mitigates environmental pollution but also promotes sustainable practices in water management.

Mechanisms of Phytorizofiltration

Plants utilize several mechanisms to filter pollutants, which include: root uptake, translocation, biochemical transformations, rhizodegradation (Juhasz, 2000).

Benefits of Phytorhizofiltration

Phytorhizofiltration offers numerous advantages for environmental remediation and water management (Figure 1.35).

1. **Cost-Effectiveness**: Compared to traditional remediation methods, such as excavation or chemical treatment, phytorhizofiltration is generally more economical. It requires less energy and infrastructure investment, making it a viable option for large-scale applications.
2. **Environmental Sustainability**: This method utilizes natural biological processes, reducing reliance on synthetic chemicals and minimizing ecological disruption. It promotes biodiversity and can enhance soil health.
3. **Versatility**: Phytorhizofiltration can be applied to a wide range of contaminants, including heavy metals, organic pollutants, and excess nutrients. This versatility allows for its use in diverse environments, from industrial sites to urban areas.
4. **Aesthetic Value**: Incorporating plants into remediation strategies can improve the aesthetic appeal of contaminated sites. Green spaces can enhance local biodiversity and provide recreational opportunities for communities.
5. **Community Engagement and Education**: Phytorhizofiltration projects can foster community involvement and raise awareness about environmental issues, promoting a sense of stewardship and responsibility.
6. **Carbon Sequestration**: The plants used in phytorhizofiltration can sequester carbon, helping to mitigate climate change while improving soil organic matter.
7. **Reduction of Water Treatment Costs**: By improving the quality of water at the source, phytorhizofiltration can alleviate the burden on water treatment facilities, leading to lower operational costs.

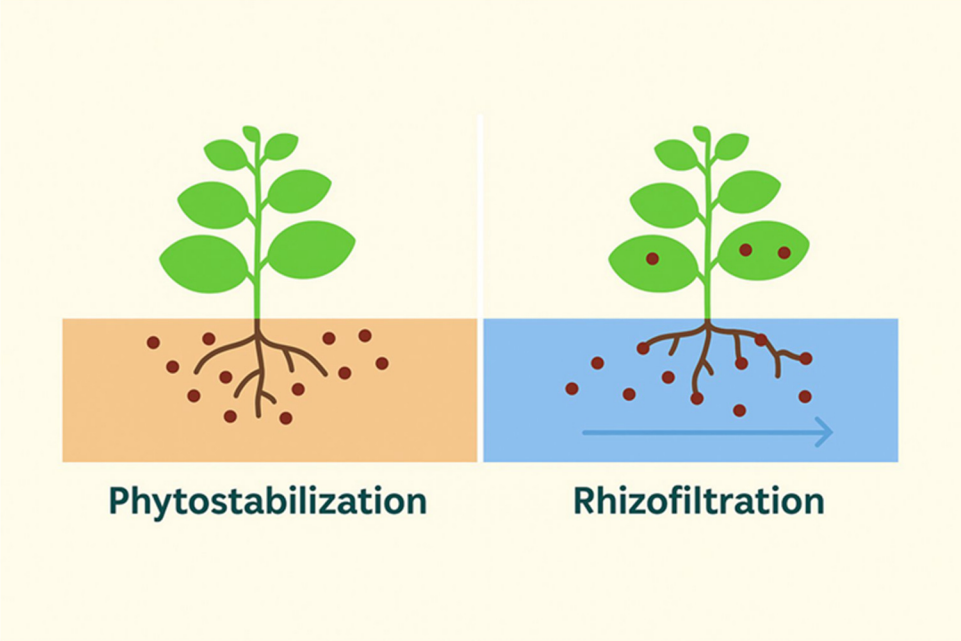

Figure 1.35 The effect of phytostabilization and rhizophyllization
Source: original

1.3 Uptake of heavy metals

There are different types of processes that enable uptake and translocation of heavy metals in plants, including uptake by the root system, root-shoot transport, xylem, and sequestration (Yan et al., 2020).

While many heavy metals exist in soluble forms and are easily absorbed by plants, there are also insoluble forms.

Plants develop different mechanisms to facilitate the availability of these metals in the soil.

One of these mechanisms involves the secretion of chelating compounds by plant roots, making them available in the soil (Dalvi and Bhalerao, 2013).

1. **Root uptake**

The first step in the uptake of heavy metals occurs at the plant roots. Heavy metals can enter the plant through two primary pathways:

- **Apoplastic Pathway**: This pathway involves passive diffusion, where heavy metals are absorbed by non-living tissues, primarily through the cell walls and intercellular spaces. The apoplastic route allows for the movement of metals without crossing the plasma membranes of root cells, facilitating quick uptake (Peer et al., 2005). However, this pathway is limited to certain metal forms and concentrations.
- **Symplastic Pathway**: In contrast, the symplastic pathway is an active transport process, where heavy metals move through living cells via the cytoplasm. This mechanism requires energy, typically derived from ATP, to transport metals across the plasma membranes. This pathway is crucial for the uptake of essential micronutrients and helps regulate metal homeostasis within the plant (Fitzgerald et al., 2019).

2. **Secretion of chelating compounds**

Plants have developed mechanisms to enhance the bioavailability of heavy metals in the soil. One key mechanism is the secretion of chelating compounds from root exudates. These compounds, which include organic acids, amino acids, and phytochelatins, bind to heavy metals, increasing their solubility and facilitating uptake (Dalvi and Bhalerao, 2013). For example, citric acid and malic acid can effectively mobilize iron and other metal ions, making them more accessible to plants (Sato et al., 2018).

3. **Complex formation and immobilization**

Once absorbed, heavy metals form complexes with various chelators in root cells. These complexes can be immobilized in extracellular spaces or within organelles, preventing the metals from exerting toxic effects on cellular functions (Ali et al., 2013). Chelation reduces the bioavailability of heavy metals, allowing plants to detoxify and store them safely. Additionally, vacuolar sequestration is a common strategy where metals are stored in vacuoles, further isolating them from metabolic pathways (Yuan et al., 2015).

4. **Transport to the xylem**

After immobilization, heavy metal ions that are sequestered in root tissues are transported to the stele, where they enter the xylem vessels. This transition is essential for upward transport, as the xylem conducts water and dissolved nutrients from the roots to the upper parts of the plant (Thakur et al., 2016). The movement through the xylem also facilitates the distribution of metals required for physiological functions in various plant tissues.

6. **Translocation through xylem**

Once in the xylem, heavy metals are translocated through the plant, reaching stems, leaves, and even fruits. This process is primarily driven by transpiration, where water evaporates from the leaf surface, creating negative pressure that pulls water (and dissolved metals) upward (Kumar et al., 2022). The efficiency of translocation can vary among different plant species, with some species demonstrating a higher capacity for metal accumulation in aerial parts, which is crucial for phytoremediation efforts.

7. **Role of mycorrhizal associations**

Many plants also form symbiotic relationships with mycorrhizal fungi, which enhance nutrient uptake, including heavy metals. Mycorrhizal fungi increase the root surface area, improving the plant's ability to absorb metals from the soil. This relationship can lead to enhanced metal uptake and translocation, as fungi can solubilize nutrients and heavy metals, making them more accessible to plant roots (Smith and Read, 2008).

PATHWAYS OF HEAVY METAL INTAKE

The intake of these heavy metals occurs through two distinct and crucial pathways: apoplastic and symplastic. The apoplastic pathway represents a passive diffusion mechanism wherein nonliving tissue, such as the cell walls and intercellular spaces, absorbs the heavy metals. This process does not require energy and allows for the movement of metal ions through the spaces between cells, effectively bypassing the cellular membranes. In contrast, the symplastic pathway involves active transport, where heavy metals penetrate living tissue, traversing through the cytoplasm and cell membranes of root cells. This pathway is energetically demanding as it utilizes ATP to

facilitate the movement of metal ions, ensuring that essential nutrients and heavy metals can be selectively taken up by the plant (Peer et al., 2005).

Upon absorption, heavy metals interact with various chelators, which are organic compounds that can form stable complexes with metal ions. These complexes are formed within root cells, serving to immobilize the heavy metals in either extracellular or intracellular spaces. This process is vital as it prevents the metals from interfering with essential cellular functions and minimizes their toxicity to the plant (Ali et al., 2013). For instance, chelators such as phytochelatins and metallothioneins play an essential role in detoxifying heavy metals and facilitating their storage in vacuoles or cell walls.

Once these heavy metal ions are sequestered within the cell space, they are then transferred to the stele, the central part of the root where vascular tissue is located. From there, they move through the xylem, a type of vascular tissue responsible for water and nutrient transport, as they journey upward through the roots towards the aerial parts of the plant (Thakur et al., 2016). The xylem vessels create a continuous network that allows for the efficient movement of water, dissolved nutrients, and heavy metals, which are essential for various physiological processes.

Finally, these heavy metals are translocated through the xylem vessels and out of the roots, spreading further into the plant's structure, including the stems and leaves (Kumar et al., 2022). This translocation process is not merely a passive occurrence; it is intricately regulated by the plant's physiological state and environmental conditions. Factors such as transpiration rates, nutrient availability, and the presence of specific metal transporters can significantly influence the extent of heavy metal uptake and distribution throughout the plant.

Understanding these pathways of heavy metal intake is critical, especially in an era where soil and water pollution are prevalent due to industrial activities, agricultural practices, and urbanization. As college students studying environmental science, biology, or related fields, it is imperative to grasp the implications of heavy metal accumulation in plants, which not only affects plant health but also poses risks to food safety and human health through the food chain. Furthermore, investigating the mechanisms underlying heavy metal uptake can aid in the development of

phytoremediation strategies, where plants are used to extract or stabilize heavy metals from contaminated environments, thereby contributing to ecological restoration and sustainability (Pietrini, 2005).

PHYTOREMEDIATION AS SOLUTION

Currently, phytoremediation has become an effective and affordable technological solution used to extract or remove inactive metals and metal contaminants from contaminated soil.

Plants with exceptional metal accumulation capacity are known as hyperaccumulator plants (Trapp and Legind 2010).

Many types of plants manage to absorb pollutants such as lead, cadmium, chromium, arsenic and various radionuclides from the soil. Thus, one of the categories of phytoremediation, phytoextraction, can be used to remove heavy metals from soil using its ability to absorb metal that are essential for plant growth (Fe, Mn, Zn, Cu, Mg, Mo and Ni) .

Some metals with an unknown biological function can also be accumulate (Cd, Cr, Pb, Co, Ag, Se, Hg) (Vamerali, et al., 2009).

Table 1.1 Types of plants and their ability to mediate heavy metals

Plant species	plant tissue	Resistant metals
Alyssum bertolonii	Leaves, stem, roots	Ni
Alnus firma	Roots	Pb, Cu
Brassica napus	Roots	Pb
Thlaspi caerulescens	Stem, root	Zn,Cd
Thlaspi goesingense	Stem	Ni
Solanum nigrum	Roots, stems &leaves	Cu, Cd, Cr
Arabis hirsute	stems &leaves	Pb, Zn
Brassica chinensis	Roots	Cd, Pb
Commelina communis	Roots,stems and leaves.	Pb
Elsholtzia splendens	Roots, stem and leaves	Cu

The advantages of phytoremediation are its efficiency in pollutant reduction, low cost, applicability for a wide range of pollutants and generally it is an environmentally friendly method.

At the same time, it is a financially acceptable and cheaper option for environmental pollution remediation and is especially suitable for large sites that have relatively low levels of contamination.

This technology has recently attracted attention as an innovative alternative to the more established treatment methods used in hazardous waste landfills because it does not require expensive equipment or highly specialized personnel.

It is also cost-effective for remediation of large amounts of water with low contaminant concentrations and for large areas with low to moderately contaminated surface soils.

REMEDIATION PLANTS IN NATURE

Various plant species have demonstrated the ability to remediate contaminated environments through mechanisms like phytoremediation, phytodegradation, and phytostabilization (Table 1).

Sunflower

- **Contaminants**: Heavy metals (lead, cadmium, uranium).
- **Mechanism**: Sunflowers are known for their ability to absorb and store heavy metals in their tissues. Studies have shown that they can effectively extract heavy metals from contaminated soils, making them valuable for bioremediation (Brown et al., 2003).

Willow

- **Contaminants**: Heavy metals and organic pollutants.
- **Mechanism**: Willows have extensive root systems that enhance the uptake of contaminants from soil and water. Their ability to support microbial activity in the rhizosphere further improves their effectiveness in phytoremediation (Huang et al., 2008).

Indian mustard

- **Contaminants**: Heavy metals (lead, cadmium, selenium).
- **Mechanism**: This species is a well-known hyperaccumulator of heavy metals. Research indicates that Indian mustard can absorb significant amounts of heavy metals, making it useful for soil remediation (Kumar et al., 2011).

Common reed

- **Contaminants**: Nutrients (nitrogen, phosphorus) and organic pollutants.
- **Mechanism**: Common reed is effective in filtering pollutants from water and improving water quality through its extensive root system. Its role in constructed wetlands highlights its utility in wastewater treatment (Vymazal, 2011).

Coriander

- **Contaminants**: Heavy metals (lead, cadmium).
- **Mechanism**: Coriander has shown potential in absorbing heavy metals from contaminated soils, making it a candidate for urban soil remediation (Jabeen et al., 2015).

These plants serve as natural tools for environmental remediation, offering a sustainable and effective means to combat soil and water contamination. Their ability to absorb, stabilize, and detoxify pollutants not only improves environmental health but also contributes to biodiversity and ecosystem restoration. Utilizing these species in remediation efforts can provide ecological benefits while addressing contamination challenges.

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CHAPTER 2. ANTIMICROBIAL RESISTANCE FROM NATURAL SELECTION EXACERBATED BY HUMAN FACTORS AND INNOVATIVE GREEN METHODS TO COMBAT IT

2.1 Introduction

The growing resistance of bacteria to antibiotics, along with the increasing frequency of unsuccessful infection treatments, highlights the urgent need to identify the underlying causes of this issue and to explore strategies for mitigating it and improving treatment outcomes. One widely recognized factor contributing to treatment failure is the selective pressure exerted by drugs, particularly when antibiotics are improperly chosen or administered at subtherapeutic doses. This can lead to the survival of resistant bacterial populations or the activation of resistance mechanisms [Cantón, 2011]. Consequently, it is crucial to use antibiotics appropriately—only in confirmed bacterial infections and at doses that maximize the likelihood of therapeutic success.

In the early stages of infection, especially in severe cases, empirical therapy is commonly initiated. This involves selecting an antibiotic based on factors such as the infection site, the patient's clinical condition, medical history, concurrent illnesses, and organ function. The chosen antibiotic should be effective against the most probable pathogens, whose prevalence and susceptibility profiles are typically known from epidemiological data derived from retrospective analyses of numerous microbiological studies. Whenever feasible, empirical antibiotic therapy should be preceded by specimen collection for microbiological testing. The results of these tests then serve to validate initial therapeutic decisions and guide the transition to targeted therapy. Thus, microbiological findings provide strong support for selecting the optimal antibiotic in both empirical and targeted treatment approaches.

Accurate pathogen identification—sometimes including quantification per gram or millilitre of sample—combined with analytical and clinical data, forms the basis for definitive infection diagnosis. Antibiograms, on the other hand, offer guidance on selecting drugs expected to be clinically effective against the identified bacteria. Typically, antibiograms include qualitative assessments of bacterial susceptibility or

resistance, along with information on detected resistance mechanisms. For many infections, such data suffice to discontinue ineffective antibiotics and replace them with agents to which the bacteria are susceptible.

However, in seriously ill patients, those with chronic infections, extensive prior antibiotic exposure, or histories of treatment failure, more precise microbiological guidance is essential to facilitate optimal antibiotic selection. Two commonly used methods to assess bacterial susceptibility are the Kirby-Bauer Disk Diffusion Susceptibility Test and the determination of the Minimum Inhibitory Concentration (MIC). The Kirby-Bauer method is widely applied as a standardized, cost-effective, and rapid qualitative test that guides initial empirical therapy. Following this, MIC testing provides a more precise, quantitative measure of antibiotic effectiveness, which is particularly valuable for tailoring therapy in complex or resistant infections. Although MIC determination has long been established, it was historically performed sporadically; however, it is now increasingly included in routine testing. Despite this, the practical use of MIC results for optimizing therapy remains limited, and sometimes, despite higher costs compared to qualitative methods, MIC testing is not fully utilized.

2.2 Types of Tests and Work Protocols

Kirby-Bauer Disk Diffusion Susceptibility Test Protocol

History

The publication on penicillin by Alexander Fleming in 1928 is a milestone in the history of medicine. As more antimicrobial compounds were discovered, it was predicted that infectious diseases would be eliminated through the use of these antimicrobials [Jorgensen et al., 2007]. Unfortunately, the development of bacterial resistance to these antimicrobials quickly diminished this optimism and resulted in the need for physicians to request the microbiology lab to test a patient's pathogen against various concentrations of a given antimicrobial to determine susceptibility or resistance to that drug. The original method of determining susceptibility to antimicrobials was based on broth dilution methods [Jorgensen et al., 2007], which although still the gold standard today, are time consuming to perform. This prompted the development of a disk diffusion procedure for the determination of susceptibility of bacteria to

antimicrobials. By the early 1950s, most clinical microbiology laboratories in the United States had adopted the disk diffusion method for determining susceptibility of bacteria to antimicrobials. Each lab modified the procedure to suit its own needs, which included using different types of media, inoculum concentration, incubation time, incubation temperature, and concentration of the antimicrobial compound. Interpretation of susceptibility and resistance was based only on the presence or absence of a zone of inhibition surrounding the disk, and two or three different concentrations of the same antimicrobial were routinely tested against the pathogen [Bauer et al., 1959].

Many researchers published variations for the procedure resulting in multiple protocols that resulted in widespread confusion [Bauer et al., 1959; Kirby et al., 1959]. In 1956, W. M. M. Kirby and his colleagues at the University of Washington School of Medicine and the King County Hospital proposed a single disk method for antimicrobial susceptibility testing [Winn et al., 2006]. The lack of standardization for the determination of bacterial susceptibility continued to be a problem throughout the early 1960s. Kirby and his colleague, A. W. Bauer, extensively reviewed the susceptibility testing literature. They consolidated and updated all the previous descriptions of the disk diffusion method and published their findings [Bauer et al., 1966]. This publication led the World Health Organization to form a committee in 1961 to lay the groundwork for the development of a standardized procedure for single antimicrobial disk susceptibility testing [Jorgensen et al., 2007].

The result was a standardized procedure for the disk diffusion susceptibility test, henceforth called the Kirby-Bauer disk diffusion test [Bauer et al., 1966]. Currently, the Clinical Laboratory Standards Institute (CLSI) is responsible for updating and modifying the original procedure of Kirby and Bauer through a global consensus process. This ensures uniformity of technique and reproducibility of results as pathogens develop new mechanisms of resistance and new antimicrobials are developed to fight these organisms. Interpretative guidelines for zone sizes are included in their publications (CLSI, 2006). The CLSI publication, Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard 9th Edition, represents the standard for clinical laboratories performing susceptibility testing today.

Purpose

The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds in order to assist a physician in selecting treatment options for his or her patients. The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism.

Theory

Determination of bacterial resistance to antimicrobials is an important part of the management of infections in patients. The disk diffusion method of Kirby and Bauer has been standardized and is a viable alternative to broth dilution methods for laboratories without the resources to utilize the newer automated methods for broth microdilution testing. When a 6-mm filter paper disk impregnated with a known concentration of an antimicrobial compound is placed on a Mueller-Hinton (MH) agar plate, immediately water is absorbed into the disk from the agar. The antimicrobial begins to diffuse into the surrounding agar. The rate of diffusion through the agar is not as rapid as the rate of extraction of the antimicrobial out of the disk, therefore the concentration of antimicrobial is highest closest to the disk and a logarithmic reduction in concentration occurs as the distance from the disk increases [Jorgensen et al., 2007].

The rate of diffusion of the antimicrobial through the agar is dependent on the diffusion and solubility properties of the drug in MH agar [Bauer et al., 1966] and the molecular weight of the antimicrobial compound. Larger molecules will diffuse at a slower rate than lower molecular weight compounds. These factors, in combination, result in each antimicrobial having a unique breakpoint zone size indicating susceptibility to that antimicrobial compound. If the agar plate has been inoculated with a suspension of the pathogen to be tested prior to the placing of disks on the agar surface, simultaneous growth of the bacteria and diffusion of the antimicrobial compounds occurs. Growth occurs in the presence of an antimicrobial compound when the bacteria reach a critical mass and can overpower the inhibitory effects of the

antimicrobial compound. The estimated time of a bacterial suspension to reach critical mass is 4 to 10 hours for most commonly recovered pathogens, but is characteristic of each species, and influenced by the media and incubation temperature [Jorgensen et al., 2007]. The size of the zone of inhibition of growth is influenced by the depth of the agar, since the antimicrobial diffuses in three dimensions, thus a shallow layer of agar will produce a larger zone of inhibition than a deeper layer. The point at which critical mass is reached is demonstrated by a sharply marginated circle of bacterial growth around the disk. The concentration of antimicrobial compound at this margin is called the critical concentration and is approximately equal to the minimum inhibitory concentration obtained in broth dilution susceptibility tests. Zone size observed in a disk diffusion test has no meaning in and of itself [Jorgensen et al., 2007]. The interpretation of resistance and susceptibility to antimicrobials is determined through in vivo testing of blood and urine to calculate the obtainable level of a given antimicrobial that results in resolution of an infection. This information is correlated with zone sizes resulting in the interpretive standards. The current interpretation standards can be found in the Clinical Laboratory Standards Institute Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standards 9th Edition [CLSI 2006].

RECIPE

Sterile saline in 2-ml tubes	18- to 24-ho
0.5 McFarland standard	Vortex
Wickerham card	Sterile swabs
Mueller-Hinton agar plates, 100 mm or 150 mm	Inoculating
Caliper or ruler Antibiotic disksb	Bact-cinerato
Forceps	Alcohol pads
Antibiotic disk dispenser (optional)	35°C to 37°C

[a] Recommended organisms for quality assurance purposes are *Staphylococcus aureus* ATCC 25923 (Biosafety level (BSL) 2), *Escherichia coli* ATCC 25922 (BSL 1), and *Pseudomonas aeruginosa* ATCC 27853 (BSL 2) (www.atcc.org), as the zone of inhibition for these organisms is known. Because the zone sizes are known for these organisms, they are recommended for use in the educational setting, although the use

of unknowns should also be incorporated into the educational experience. For quality control testing, the zone sizes for these three organisms can be found on the package insert from any antimicrobial disk you purchase.

[b] Selection of antimicrobial is based on the type of organism being tested and source of the isolate (blood, urine, wound, etc.). See the Interpretative Standards Tables for suggested antimicrobials to use in this exercise.

Additional Notes

Mueller-Hinton agar

MH agar is considered the best medium to use for routine susceptibility testing of no fastidious bacteria for the following reasons:

- It shows acceptable batch-to-batch reproducibility for susceptibility testing
- It is low in sulphonamide, trimethoprim, and tetracycline inhibitors
- It supports satisfactory growth of most no fastidious pathogens
- A large body of data and experience has been collected concerning susceptibility tests performed with this medium [Winn et al., 2006].

Please note that the use of media other than Mueller-Hinton agar may result in erroneous results. Also note that only the aerobic or facultative bacteria that grow well on unsupplemented MH agar should be tested using this protocol. Fastidious organisms require MH agar supplemented with additional nutrients and require that modification to this protocol be made. Neither the supplements nor the procedural modification are discussed in this basic protocol.

MH agar may be purchased as prepared agar plates
from Remel (Lenexa, KS), BD BBL (Franklin Lakes, NJ), or any other supplier of prepared agar plates. Follow the manufacturer's recommendation for storage of prepared plates. MH agar can also be prepared from dehydrated media available from companies such as Remel, BD BBL, or any other supplier of dehydrated media. Be sure to prepare the media according to the manufacturer's directions.

Formula for Mueller-Hinton agar per liter of purified water [DIFCO 1984]

Beef, Infusion from	300.0 g
Casamino acid, technical	17.5 g
Starch	1.5 g
Agar	17.0 g

Suspend the components listed above in 1 litter of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the components. Autoclave at 121°C for 15 minutes. Dispense as desired. Allow to solidify at room temperature, then store at 4 to 8°C. Mueller-Hinton agar is stable for approximately 70 days (per Remel Technical Services, 1 September 2009) from the date of preparation. Each lab should verify the quality and functionality of each batch of prepared media by testing known strains of organisms against each antimicrobial compound being used as the 70-day expiration date approaches.

- If you prepare the MH agar plates from dehydrated media, the plates must be poured to a depth of 4 mm (approximately 25 ml of liquid agar for 100-mm plates and 60 ml of liquid agar for 150-mm plates, but in any case to a measured depth of 4 mm). Plates that are too shallow will produce false susceptible results as the antimicrobial compound will diffuse further than it should, creating larger zones of inhibition. Conversely, plates poured to a depth >4 mm will result in false resistant results.
- pH of the MH agar should fall between 7.2 and 7.4 at room temperature after solidification and should be tested when the media is first prepared. If the pH is 7.4, the opposite results may occur.
- Excessive thymidine or thymine can reverse the inhibitory effects of sulphonamides and trimethoprim resulting in smaller and less distinct zones of inhibition, or no zones at all.
- The incorrect concentration of divalent cations (calcium and magnesium) will affect the results of aminoglycoside and tetracycline tests against Pseudomonas aeruginosa. Excess cation concentration will result in reduced zone sizes and low concentration will increase zone sizes. Excess calcium will increase the zone size

of *P. aeruginosa* against daptomycin. Excess zinc ions may reduce the zone size of carbapenems against *P. aeruginosa*.

- MH agar should be tested with known strains of organism at least weekly in order to verify that the media and disks are working as expected.

Antibiotic susceptibility disks

Antimicrobial disks can be purchased from any reputable suppliers, such as Remel, Oxoid or BD BBL. They are packaged in spring-loaded cartridges containing 25 or 50 disks and can be ordered as individual cartridges or in packages of 10 cartridges. Proper storage of these disks is essential for reproducible results.

Sealed cartridges containing commercially prepared paper disks should be stored at either 8°C or frozen at -14°C in a non-self-defrosting freezer. Allow disks to come to room temperature prior to removing the protective plastic packaging. Once opened, store the cartridges in a storage container containing desiccant for no more than 1 week.

Semiautomatic disk dispensers are available from companies such as Remel, Oxoid and BD BBL. Be aware that disk cartridges from one company may not fit the dispenser of another company.

Figure 2.1 Examples of antibiotics

Semiautomatic disk dispensers are available from companies such as Remel, Oxoid and BD BBL. Be aware that disk cartridges from one company may not fit the dispenser of another company.

Figure 2.2 Semiautomatic disk dispensers

McFarland standard

McFarland standards are suspensions of either barium sulphate or latex particles that allow visual comparison of bacterial density. Commercially prepared standards are available for purchase from companies such as Remel or BD BBL. These often include a Wickerham card, which is a small card containing parallel black lines. A 0.5 McFarland standard is equivalent to a bacterial suspension containing between 1 x 108 and 2 x 108 CFU/ml of *E. coli*.

A 0.5 McFarland standard may be prepared in-house as describe below.

1. Add a 0.5-ml aliquot of a 0.048 mol/liter $BaCl_2$ (1.175% wt/vol $BaCl^2 \cdot 2H_20$) to 99.5 ml of 0.18 mol/litter H_2SO_4 (1% vol/vol) with constant stirring to maintain a suspension.

2. Verify the correct density of the turbidity standard by measuring absorbance using a spectrophotometer with a 1-cm light path and matched cuvette. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.

3. Transfer the barium sulphate suspension in 4- to 6-ml aliquots into screw-cap tubes of the same size as those used in standardizing the bacterial inoculums.

4. Tightly seal the tubes and store in the dark at room temperature.

Use of the McFarland standard in the Kirby-Bauer procedure.

1. Prior to use, vigorously agitate the barium sulphate standard on a mechanical vortex mixer and inspect for a uniformly turbid appearance. Replace the standard if large particles appear. If using a standard composed of latex particles, mix by inverting gently, not on a vortex mixer.
2. As the student adds bacterial colonies to the saline in the “preparation of the inoculum” step of the procedure, he or she should compare the resulting suspension to the McFarland standard. This is done by holding both the standard and the inoculum tube side by side and no more than 1 inch from the face of the Wickerham card (with adequate light present) and comparing the appearance of the lines through both suspensions. Do not hold the tubes flush against the card. If the bacterial suspension appears lighter than the 0.5 McFarland standard, more organisms should be added to the tube from the culture plate. If the suspension appears more dense than the 0.5 McFarland standard, additional saline should be added to the inoculum tube in order to dilute the suspension to the appropriate density. In some cases it may be easier to start over rather than to continue to dilute a bacterial suspension that is too dense for use.

Figure 2.3 Density measurement

PROTOCOL

Preparation of Mueller-Hinton plate

1. Allow a MH agar plate (one for each organism to be tested) to come to room temperature. It is preferable to allow the plates to remain in the plastic sleeve while they warm to minimize condensation.

2. If the surface of the agar has visible liquid present, set the plate inverted, ajar on its lid to allow the excess liquid to drain from the agar surface and evaporate. Plates may be placed in a 35°C incubator or in a laminar flow hood at room temperature until dry (usually 10 to 30 minutes).

3. Appropriately label each MH agar plate for each organism to be tested.

Preparation of inoculum

1. Using a sterile inoculating loop or needle, touch four or five isolated colonies of the organism to be tested.
2. Suspend the organism in 2 ml of sterile saline.

3. Vortex the saline tube to create a smooth suspension.
4. Adjust the turbidity of this suspension to a 0.5 McFarland standard by adding more organism if the suspension is too light or diluting with sterile saline if the suspension is too heavy.
5. Use this suspension within 15 minutes of preparation.

Additional Notes

Inoculum preparation

Organisms to be tested must be in the log phase of growth in order for results to be valid. It is recommended that subcultures of the organisms to be tested be made the previous day.

Never use extremes in inoculum density. Never use undiluted overnight broth cultures or other unstandardized inocula for inoculating plates.

If the organism is difficult to suspend directly into a smooth suspension, the growth method of preparing the inoculums should be used. However, the recommended organisms listed in this procedure all produce smooth suspensions with little difficulty. See the Clincial Laboratory Standards Institute document [CLSI 2006] for the growth procedure method for preparing the inoculums, if needed.

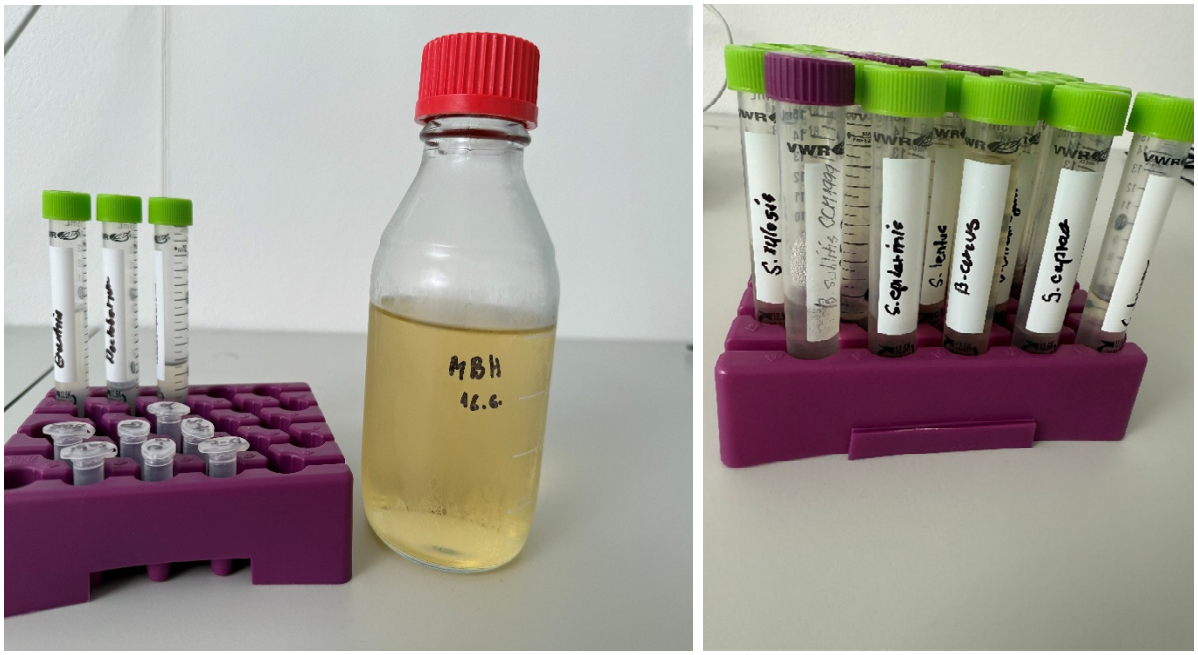

Figure 2.4 MH agar, bacterial suspension

Inoculation of the MH plate

1. Dip a sterile swab into the inoculum tube.
2. Rotate the swab against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid. The swab should not be dripping wet.
3. Inoculate the dried surface of a MH agar plate by streaking the swab three times over the entire agar surface; rotate the plate approximately 60 degrees each time to ensure an even distribution of the inoculum).
4. Rim the plate with the swab to pick up any excess liquid).
5. Discard the swab into an appropriate container.
6. Leaving the lid slightly ajar, allow the plate to sit at room temperature at least 3 to 5 minutes, but no more than 15 minutes, for the surface of the agar plate to dry before proceeding to the next step.

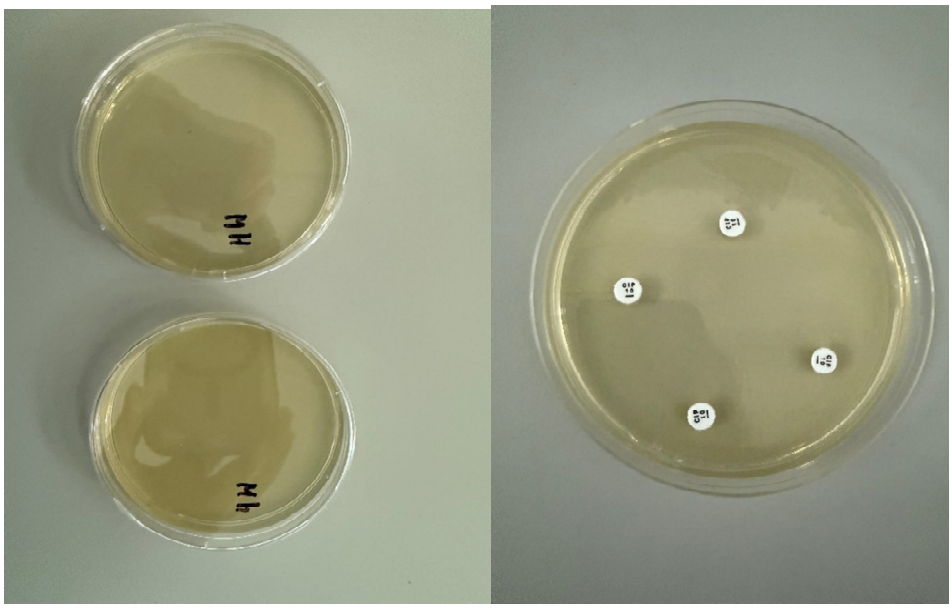

Figure 2.5 MH plates, ATB application

Placement of the antibiotic disks

1. Place the appropriate antimicrobial-impregnated disks on the surface of the agar, using either forceps to dispense each antimicrobial disk one at a time, or a multidisk dispenser to dispense multiple disks at one time. (See steps a. through d. for the use of the multi-disk dispenser or steps e. through g. for individual disk placement with forceps.

a. To use a multidisc dispenser, place the inoculated MH agar plate on a flat surface and remove the lid.

b. Place the dispenser over the agar plate and firmly press the plunger once to dispense the disks onto the surface of the plate.

c. Lift the dispenser off the plate and using forceps sterilized by either cleaning them with an alcohol pad or flaming them with isopropyl alcohol, touch each disk on the plate to ensure complete contact with the agar surface. This should be done before replacing the petri dish lid as static electricity may cause the disks to relocate themselves on the agar surface or adhere to the lid.

d. Do not move a disk once it has contacted the agar surface even if the disk is not in the proper location, because some of the drug begins to diffuse immediately upon contact with the agar.

e. To add disks one at a time to the agar plate using forceps, place the MH plate on the template provided in this procedure. Sterilize the forceps by cleaning them with a sterile alcohol pad and allowing them to air dry or immersing the forceps in alcohol then igniting.

f. Using the forceps carefully remove one disk from the cartridge.

g. Partially remove the lid of the petri dish. Place the disk on the plate over one of the dark spots on the template and gently press the disk with the forceps to ensure complete contact with the agar surface. Replace the lid to minimize exposure of the agar surface to room air.

h. Continue to place one disk at a time onto the agar surface until all disks have been placed as directed in steps f. and g. above.

2. Once all disks are in place, replace the lid, invert the plates, and place them in a 35°C air incubator for 16 to 18 hours. When testing *Staphylococcus* against oxacillin or vancomycin, or *Enterococcus* against vancomycin, incubate for a full 24 hours before reading.

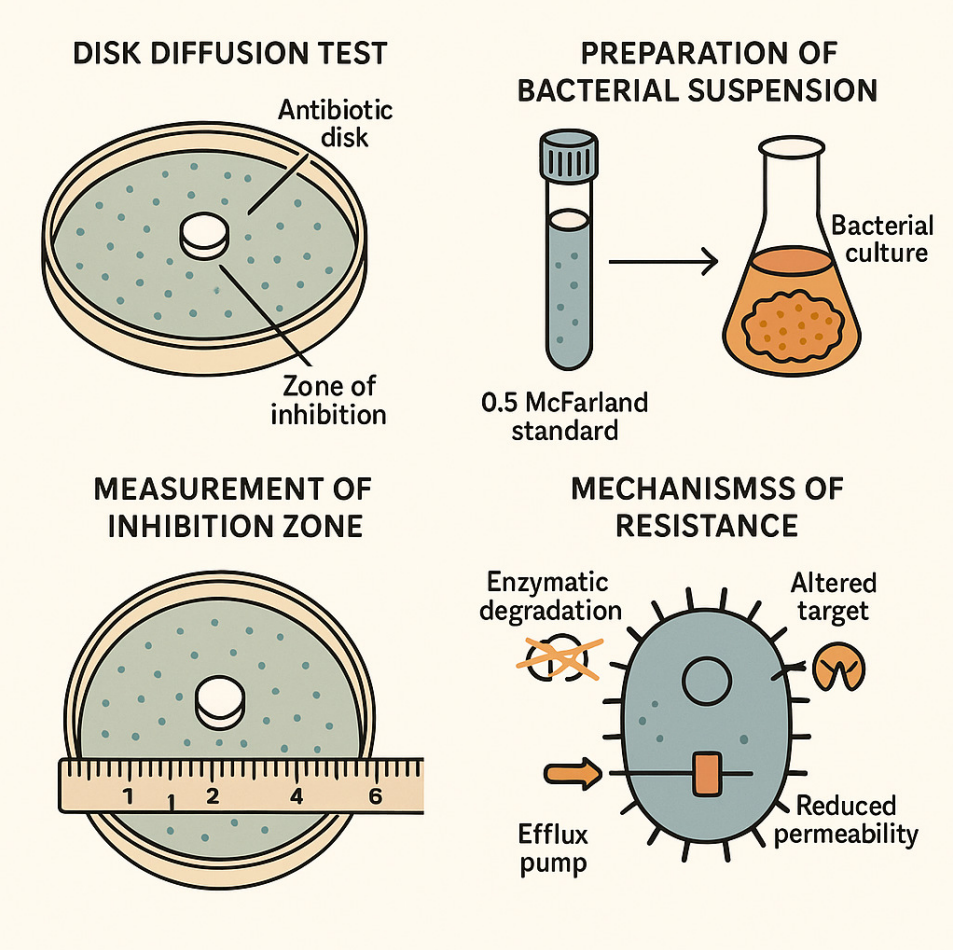

Figure 2.6 Schematic of disc diffusion method

Additional Notes

Disk placement

Disks should not be placed closer than 24 mm (centre to centre) on the MH agar plate. Ordinarily, no more than 12 disks should be placed on a 150-mm plate or more than 5 disks on a 100-mm plate. However, the semiautomatic disk dispensers hold 16 and 8 disks respectively and may not maintain the recommended 24 mm centre to centre spacing. The template provided in this protocol maintains the recommended 24 mm centre to centre spacing and allows the placement of up to 8 disks on the plate.

You should avoid placing disks close to the edge of the plate as the zones will not be fully round and can be difficult to measure.

Each disk must be pressed down with forceps to ensure complete contact with the agar surface or irregular zone shapes may occur.

If the surface of the agar is disrupted in any way (a disk penetrating the surface, visible lines present due to excessive pressure of the swab against the plate during inoculation, etc.) the shape of the zone may be affected.

When printing the template for use in your microbiology lab, be sure that the diameter of the circle on the template is the same size as the Mueller-Hinton agar plates that you use in lab (100 mm). The "reduce" or "enlarge" function on a photocopier can be used to change the size of the template if needed. You may also make your own template by drawing a circle around a MH agar plate on a sheet of paper. Add the placement marks based on the number of disks you plan to use in your lab session, maintaining the recommended spacing as indicated above.

Incubation of the plates

A temperature range of 35°C ± 2°C is required.

Note that temperatures above 35°C may not allow the detection of methicillin-resistant Staphylococcus.

Do not incubate plates in CO_2 as this will decrease the pH of the agar and result in errors due to incorrect pH of the media.

Results can be read after 18 hours of incubation unless you are testing Staphylococcus against oxacillin or vancomycin, or Enterococcus against vancomycin. Read the results for the other antimicrobial disks then re-incubate the plate for a total of 24 hours before reporting vancomycin or oxacillin.

Measuring zone sizes

1. Following incubation, measure the zone sizes to the nearest millimetre using a ruler or calliper; include the diameter of the disk in the measurement.

2. When measuring zone diameters, always round up to the next millimetre.

3. All measurements are made with the unaided eye while viewing the back of the petri dish. Hold the plate a few inches above a black, non-reflecting surface illuminated with reflected light.

4. View the plate using a direct, vertical line of sight to avoid any parallax that may result in misreading.

5. Record the zone size on the recording sheet.

6. If the placement of the disk or the size of the zone does not allow you to read the diameter of the zone, measure from the centre of the disk to a point on the circumference of the zone where a distinct edge is present (the radius) and multiply the measurement by 2 to determine the diameter.

7. Growth up to the edge of the disk can be reported as a zone of 0 mm.

8. Organisms such as Proteus mirabilis, which swarm, must be measured differently than no swarming organisms. Ignore the thin veil of swarming and measure the outer margin in an otherwise obvious zone of inhibition.

9. Distinct, discrete colonies within an obvious zone of inhibition should not be considered swarming. These colonies are either mutant organisms that are more resistant to the drug being tested, or the culture was not pure and they are a different organism. If it is determined by repeat testing that the phenomenon repeats itself, the organism must be considered resistant to that drug.

Measuring zone sizes

If the plate was properly inoculated and all other conditions were correct, the zones of inhibition should be uniformly circular and there will be a confluent lawn of growth.
If individual colonies are apparent across the plate, the inoculum was too light and the test must be repeated.

The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Do not use a magnification device to observe zone edges.

When measuring the zone of inhibition for organisms that swarm (e.g., Proteus sp.), ignore the thin veil of swarming growth in an otherwise obvious zone of inhibition.

With trimethoprim and the sulphonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth) and measure the more obvious margin to determine the zone diameter [CLSI, 2006].

Practical Tips and Safety Measures

Practical Tips for Beginners

- **Contamination:** Always ensure sterility of tools, media, and work surfaces. Contamination can cause unclear or inaccurate results, such as unexpected growth within inhibition zones or outside inoculation areas. Work in a laminar flow hood whenever possible and use sterile pipettes and gloves.
- **Inoculum Preparation:** Incorrect inoculum density leads to erroneous interpretation. Too dense inoculum can reduce inhibition zones, while too dilute inoculum may enlarge them. Always compare the bacterial suspension to the 0.5 McFarland standard and adjust as needed.
- **Storage of Antibiotic Disks:** Store disks in their original packaging at 2–8 °C in a dry environment. Avoid repeated freezing and thawing. Improper storage can cause loss of antibiotic potency and inaccurate test results.
- **Signs of Invalid Tests:**

✓ Uneven bacterial distribution on agar (visible clumps or gaps).

- ✓ Inhibition zones that are unclear, fuzzy, or irregularly shaped.
- ✓ Changes in media color after inoculation or incubation.
- ✓ Disks not adhering properly to agar surface or shifting position.

Safety Measures

- **Biosafety Levels (BSL):** Determine necessary biosafety level depending on the microorganism (BSL-1 to BSL-3). Use protective clothing, gloves, and respirators as required by regulations when handling pathogenic bacteria.
- **Handling Infectious Materials:** Manipulate all samples and cultures with maximum caution to prevent exposure or spread of pathogens.
- **Waste Disposal:** Dispose of used pipettes, inoculation loops, plates, and contaminated materials according to institutional protocols, typically by autoclaving or chemical disinfection.
- **Personal Protective Equipment (PPE):** Always wear gloves, safety goggles, and lab coats. For aerosol-generating procedures or working with high-risk pathogens, consider face shields and work inside biosafety cabinets.

Interpretation and Reporting of the Results

The accurate interpretation of antibiotic susceptibility testing results is critical for guiding effective antimicrobial therapy. Both Kirby-Bauer disk diffusion and MIC determination provide essential information on the susceptibility or resistance of bacterial pathogens to antibiotics. Proper reading and reporting ensure that clinicians select the most appropriate treatment, minimizing therapeutic failures and reducing the development of resistance.

1. Use of CLSI Guidelines

Using the published CLSI guidelines, determine the susceptibility or resistance of the organism to each drug tested (see Tables 1, 2, and 3). These guidelines provide specific breakpoint values (zone diameters for disk diffusion and MIC values for broth dilution) for various bacterial species and antimicrobial agents. Note that different organisms require different interpretative criteria, so it is essential to refer to the correct chart for each tested pathogen. The abbreviated charts provided are tailored for the

author's suggested organisms and antimicrobial disks suitable for educational and routine use.

2. Recording Results

For each antimicrobial agent tested, indicate on the recording sheet whether the measured inhibition zone size or MIC value classifies the isolate as susceptible (S), intermediate (I), or resistant (R). This classification simplifies communication and decision-making, ensuring clear guidance for clinical treatment. Accurate recording of these categories is vital for reliable data interpretation and further epidemiological analysis.

3. Reporting to Clinicians

Only the qualitative interpretation categories (S, I, R) from the Kirby-Bauer disk diffusion test are typically reported to physicians, not the raw zone diameter measurements. This practice prevents misinterpretation of raw data and facilitates standardized therapeutic decisions. The MIC values, while more quantitative, are also interpreted according to breakpoints and reported in the same qualitative format for clinical relevance.

4. Recommended Antimicrobial Disks and Zone Sizes

A suggested battery of antibiotic disks is provided for educational settings to minimize the variety of antimicrobials required for testing diverse bacterial groups. Selecting appropriate disks according to the CLSI recommendations ensures reproducibility and relevance of results. Educators and laboratory personnel may modify the disk selection based on local epidemiology, availability, and specific clinical needs. Consistent use of recommended disks supports comparability of results across laboratories and over time.

Table 2.1 Zone diameter interpretative standards for *Staphylococcus* species [CLSI 2006]

***Staphylococcus* species (zone diameter, nearest whole mm)**

	Resistant	Intermediate	Susceptible
Cefazolin (30 µg)	≤ 14	15-17	≥ 18
Clindamycin (2 µg)	≤ 14	15-20	≥ 21
Erythromycin (15 µg)	≤ 13	14-22	≥ 23
Gentamicin (10 µg)	≤ 12	13-14	≥ 15
Oxacillin (1 µg)	≤ 10	11-12	≥ 13
Penicillin G (10 µg)	≤ 28	--	≥ 29
Tobramycin (10 µg)	≤ 12	13-14	≥ 15
Vancomycin (30 µg)	--	--	≥ 15

Table 2.2 Zone diameter interpretative standards for Pseudomonas aeruginosa and other non-fermenting gram-negative rods [CLSI 2006]

***Pseudomonas aeruginosa* and other non-fermentating gram negative rods (zone diameter, nearest whole mm)**

	Resistant	Intermediate	Susceptible
Amikacin (30 µg)	≤ 14	15-16	≥ 17
Cefoperazone (75 µg)	≤ 15	16.20	≥ 21
Cefotaxime (30 µg)	≤ 14	15-22	≥ 23
Gentamicin (10 µg)	≤ 12	13-14	≥ 15
Piperacillin (100 µg)	≤ 17	..	≥ 18
Tetracycline (30 µg)	≤ 14	15-18	≥ 19
Ticarcillin (75 µg)	≤ 14	--	≥ 15
Tobramycin (10 µg)	≤ 12	13-14	≥ 15

Table 2.3 Zone diameter interpretative standards for *E. coli* and other enteric gram-negative rods [CLSI 2006]

***Escherichia coli* and other gram-negative rods (zone diameter, nearest whole mm)**

	Resistant	Intermediate	Susceptible
Amikacin (30 µg)	≤ 14	15-16	≥ 17
Ampicillin (10 µg)	≤ 13	14-16	≥ 17
Cefazolin (30 µg)	≤ 14	15-17	≥ 18
Gentamicin (10 µg)	≤ 12	13-14	≥ 15
Tetracycline (30 µg)	≤ 14	15-18	≥ 19
Ticarcillin (75 µg)	≤ 14	15-19	≥ 20
Trimethoprim (5 µg)	≤ 10	11-15	≥ 16
Tobramycin (10 µg)	≤ 12	13-14	≥ 15

Interpretation of Kirby-Bauer Disk Diffusion Test Results

The Kirby-Bauer disk diffusion method determines bacterial susceptibility by measuring the diameter of the inhibition zone around antibiotic disks placed on agar plates inoculated with the test organism.

Measurement:

After incubation (typically 16–18 hours at 35 ± 2°C), use a ruler or caliper to measure the diameter of the clear zone where bacterial growth has been inhibited, in millimeters (mm). Measure from edge to edge of the clear area, including the disk diameter.

Correlation with Susceptibility:

The size of the inhibition zone reflects the effectiveness of the antibiotic against the tested organism. Larger zones generally indicate greater susceptibility, while smaller or absent zones indicate resistance.

Use of Breakpoints:

Measured zone diameters must be compared against standardized interpretative criteria provided by organizations such as CLSI or EUCAST. These breakpoints are specific to bacterial species and antibiotics.

Categorization:

Based on zone size, bacteria are categorized as:

- **Susceptible (S):** The pathogen is likely inhibited by achievable antibiotic concentrations using standard dosing.
- **Intermediate (I):** The pathogen may be inhibited if higher doses or increased exposure is possible, or if the antibiotic concentrates at the infection site.
- **Resistant (R):** The pathogen is unlikely to be inhibited by the antibiotic at achievable concentrations, and alternative therapy should be considered.

Factors Affecting Zone Size:

Zone sizes can be influenced by agar depth, inoculum density, incubation conditions, and antibiotic diffusion properties. Strict adherence to standardized testing protocols is essential for reliable results.

Example:

For *Escherichia coli* tested against ciprofloxacin:

- Zone diameter ≥ 21 mm = Susceptible
- Zone diameter 16–20 mm = Intermediate
- Zone diameter ≤ 15 mm = Resistant

A zone measurement of 23 mm would classify the isolate as susceptible.

Practical Tips for Beginners

When performing antibiotic susceptibility testing, beginners often encounter some common pitfalls. Being aware of these can improve the reliability and accuracy of results:

- **Contamination:** Always work in a clean environment and use sterile tools to prevent contamination from environmental microbes. Contaminants can lead to unexpected growth and misinterpretation of results.
- **Incorrect inoculum preparation:** The density of the bacterial suspension must match the 0.5 McFarland standard closely. Too dense inocula can lead to falsely reduced inhibition zones or higher MICs; too dilute suspensions can give falsely increased susceptibility. Always verify turbidity using a McFarland standard or spectrophotometer.
- **Improper disk storage:** Antibiotic disks must be stored according to manufacturer instructions, usually refrigerated and protected from moisture and light. Expired or improperly stored disks can lose potency, affecting test outcomes.
- **Uneven inoculum spreading:** Ensure uniform spreading of the inoculum on agar plates. Uneven lawns can produce irregular inhibition zones that are hard to measure accurately.
- **Inconsistent incubation conditions:** Maintain precise temperature (usually 35±1°C) and incubation time as per protocol. Deviations can alter bacterial growth and antibiotic activity.

Signs of Invalid Tests

Be alert to indicators suggesting the test may be invalid:

- **Diffuse or scattered bacterial growth:** Instead of a uniform lawn, scattered colonies may indicate poor inoculum preparation or contamination.
- **Unclear or fuzzy inhibition zones:** Difficult-to-define zone edges can complicate interpretation. This may result from inappropriate agar depth, incubation conditions, or suboptimal antibiotic disk quality.
- **Unusual agar color changes:** Discoloration or unexpected media color may indicate contamination or media degradation.

Minimum inhibitory concentration MIC

The minimum inhibitory concentration (MIC) refers to the lowest amount of an antibacterial substance, measured in mg/L (or µg/mL), that completely halts the visible growth of a bacterial strain under rigorously controlled laboratory (*in vitro*) conditions [EUCAST, 1998].

Methods for Determining MIC

The following approaches are commonly employed:

- **Dilution Techniques:**
  - On solid agar media
  - In liquid culture media
  - Microdilution (micro method)
  - Microdilution (macro method)
- **Gradient Techniques:**
  - Use of strips impregnated with a known antibiotic concentration gradient

Dilution Techniques

According to EUCAST [2020], broth microdilution is generally recommended, except for fosfomycin and mecillinam, for which agar dilution is preferred. Conversely, the American CLSI [2018] guidelines allow for either broth or agar dilution methods to be used interchangeably for most bacterial species and antibiotics. However, specific exceptions exist: *Haemophilus influenzae* strains and antibiotics such as colistin and

daptomycin require broth dilution exclusively for MIC determination. Fosfomycin, in line with EUCAST guidance, is evaluated by agar dilution. Additionally, CLSI advises the use of HTM medium for *H. influenzae* instead of the MH–F broth recommended by EUCAST.

For MIC testing, all quantitative procedures utilize Mueller–Hinton (MH) medium, either as agar plates (MHA) or broth (MHB). Sometimes, the medium is supplemented with additives such as 5% lysed horse blood or other components depending on the bacterial species or antibiotic class. For anaerobic bacteria, Brucella agar enriched with Hemin (5 µg/mL), Vitamin K (1 µg/mL), and 5% lysed horse blood is used [CLSI, 2018; Nagayama et al., 2008].

Working solutions of antibiotics should be prepared as twofold serial dilutions, with the concentration range selected based on the specific drug and taking into account established MIC breakpoints for reference strains [ISO 20776-1, 2016]. The serial dilutions should follow protocols outlined in the relevant guidelines [ISO 20776-1, 2016] and those recommended by EUCAST [2000].

In the broth microdilution method, these twofold diluted antibiotic solutions are dispensed into the wells of microtiter plates. These plates can be used immediately for MIC testing or stored in sealed plastic bags at temperatures of –60 °C or lower for up to three months [ISO 20776-1, 2016]. An exception is tigecycline, for which MIC testing must be performed within 12 hours of preparing the Mueller-Hinton broth (MHB) medium, due to oxygen accumulation over time that decreases tigecycline activity [EUCAST, 1998, 2000, 2020; Wiegand et al., 2008].

For the agar dilution method, 1 mL of each antibiotic dilution is added to 19 mL of molten Mueller-Hinton agar (MHA) maintained at 45–50 °C, then poured into 9-cm Petri dishes [EUCAST, 2000].

Bacterial Inoculum

The bacterial suspension used for testing should be prepared from morphologically similar colonies grown overnight on a nonselective medium, either solid or liquid. The target inoculum sizes for each method are:

- Broth microdilution: 5 × 10^5 CFU/mL [ISO 20776-1, 2016]

- Agar dilution: 1 × 10^4 CFU per spot [EUCAST, 2000, Andrews, 2001]

To prepare these suspensions, a 0.5 McFarland standard suspension is first made.

For broth microdilution, the 0.5 McFarland suspension is diluted 1:100 to approximately 1 × 10^6 CFU/mL by mixing 9.9 mL of broth with 0.1 mL of the 0.5 McFarland suspension. From this, 50 µL is added to wells containing 50 µL of the antibiotic solution, or alternatively, 10 µL of inoculum is added to 100 µL of diluted antibiotic. When commercial freeze-dried antibiotic plates are used, 50 µL of 0.5 McFarland suspension is added to 10 mL of broth to achieve the desired inoculum. For *Streptococcus pneumoniae*, 100 µL of 0.5 McFarland suspension is transferred to 10 mL broth to reach 5 × 10^5 CFU/mL [EUCAST, 2003].

In the agar dilution method, the 0.5 McFarland suspension is diluted 1:10 in saline or broth, and 1 µL of this dilution is spotted onto agar plates containing serial antibiotic dilutions [EUCAST, 2000].

Inoculation should occur within 30 minutes of preparation to maintain cell viability. Plates and broth cultures are incubated at 35 ± 1 °C for 18–24 hours under aerobic conditions. Longer incubation (24 h) is recommended for glycopeptides, oxacillin, *Streptococcus spp.*, and *Haemophilus spp.* strains [EUCAST, 2003]. Exceptions include *Neisseria spp.*, which require 5% CO_2 atmosphere, and anaerobes, which require anaerobic conditions for up to 48 hours [EUCAST, 2020].

Quality Control

Proper inoculum preparation critically affects MIC test reliability. The turbidity of the 0.5 McFarland suspension is verified by spectrophotometric absorbance at 625 nm, which should be between 0.08 and 0.13 [EUCAST, 2020; Wiegand et al., 2008]. Additionally, inoculum density in microtiter wells is checked by plating 10 µL from the growth control well onto solid media and incubating; growth of 20–80 colonies confirms the target density of 5 × 10^5 CFU/mL [ISO 20776-1, 2016].

Quality control measures also include verifying media sterility, strain viability, and reference strain MIC values, as specified by EUCAST and CLSI guidelines [EUCAST, 2020; CLSI, 2018]. MIC values for reference strains must fall within accepted ranges to validate test results.

Reading Results

The MIC is defined as the lowest antibiotic concentration that completely inhibits visible bacterial growth. In agar dilution, minimal growth such as 1–2 colonies or faint haze is disregarded [Andrews, 2001; EUCAST, 2000].

In broth microdilution, special reading criteria apply for some antibiotics. For bacteriostatic agents (e.g., chloramphenicol, tetracycline, clindamycin) against Gram-positive bacteria and for tigecycline or eravacycline against Gram-negative bacteria, pinpoint growth at the bottom of wells is ignored. For trimethoprim-sulfamethoxazole, MIC is read at the lowest concentration inhibiting ≥80% of growth compared to control [EUCAST, 2020].

Resazurin dye may be used to facilitate reading; it changes color in response to bacterial metabolic activity [Elshikh et al., 2016]. Tests with inconsistent growth patterns require repetition.

Eagle Effect

The Eagle effect describes a paradoxical increase in bacterial survival at higher-than-optimal bactericidal antibiotic concentrations, first reported for penicillin by Harry Eagle in 1948 [Jarrad et al., 2018; Prasetyoputri et al., 2019]. This phenomenon has been observed for multiple antibiotic classes and various bacterial species and can lead to treatment failure due to overdosing. Mechanisms are not fully understood but may involve increased beta-lactamase production, reduced penicillin-binding protein expression, or oxidative stress [McKay et al., 2009].

Gradient Method

The gradient method uses antibiotic-impregnated strips with predefined concentration gradients (E-test) to simplify MIC determination. While fast and easy, its use for colistin and vancomycin susceptibility testing has declined due to unreliable results [EUCAST, 2020; CLSI, 2018]. Colistin MIC values are often underestimated by gradient tests because of poor diffusion and interaction with strip materials [Karvanen et al., 2017; *Satlina, 2019*]. Similar concerns exist for fosfomycin MIC testing.

Regulatory agencies have restricted gradient methods for certain antibiotics, though research continues to improve their reliability, including calcium supplementation of media [*Gwozdzinski et al., 2018;* Green et al., 2020].

Interpretation of MIC

MIC values are compared against clinical breakpoints from organizations like EUCAST and CLSI to categorize strains as susceptible, intermediate, or resistant [EUCAST, 2020; CLSI, 2018]. These breakpoints consider pharmacological, microbiological, and clinical data and are periodically updated [Kahlmeter et al., 2014, 2015]. MIC interpretation guides clinical therapy decisions and informs further resistance mechanism investigations.

In contrast to qualitative methods, the MIC value enables assessment of the degree of bacterial susceptibility or resistance to an antibiotic. This information holds significant epidemiological and clinical importance but requires careful interpretation. Directly comparing MIC values across different antibiotics to determine which one a strain is most sensitive to is misleading. Such a practice incorrectly assumes that the antibiotic with the lowest MIC is always the most effective.

EUCAST has improved susceptibility assessment by introducing new interpretation criteria that differentiate two levels of susceptibility. The first corresponds to standard dosing regimens, while the second applies to higher MIC values requiring increased antibiotic exposure. The MIC magnitude influences pharmacokinetic/ pharmacodynamic (PK/PD) indices crucial for therapy success. It is important to note that strains with MICs near breakpoints, though classified as susceptible at standard doses, may signal potential therapy failure and emerging resistance. For example, *Salmonella enterica* serovar Typhi strains with MIC ≤ 0.06 mg/L possess a mutation in the gyrA gene linked to fluoroquinolone resistance development [Jorgensen et al., 2015; Ezadi et al., 2019]. Similarly, vancomycin MICs of 2 mg/L in *S. aureus* infections are associated with increased therapy failure risk despite susceptibility classification [van Hal et al., 2012].

Why is the degree of susceptibility important? The more susceptible a strain, the greater the likelihood its MIC falls below the epidemiological cutoff value (ECOFF),

indicating absence of resistant subpopulations and reducing treatment failure risk. High susceptibility also improves the chances of achieving therapeutic antibiotic concentrations even in patients with altered PK parameters. Knowledge of susceptibility degrees within hospitals supports antibiotic stewardship by identifying drugs with MIC90 values close to breakpoints, which may warrant restricted empirical use to minimize resistance selection [Doron et al., 2011; Kuti, 2016; Morency-Potvin et al., 2017; Mölstad et al., 2017].

Unfortunately, many hospitals lack cumulative MIC distribution data because actual MIC values (rather than automated system approximations) are seldom routinely measured, and only for selected antibiotics. Nonetheless, stewardship teams can collaborate with microbiology labs to monitor susceptibility trends, especially where therapeutic failures are frequent despite susceptibility results. Such data help tailor empirical therapy lists and dosing regimens. For instance, Kuti et al. implemented prolonged meropenem and continuous cefepime infusions in an ICU based on high MICs, enhancing treatment outcomes [Kuti, 2016]. They also incorporated linezolid for MRSA strains with MICs of 1.5–2 mg/L after failed high-dose vancomycin therapy, reducing mortality and hospital stay [Kuti, 2016]. Transferring such approaches without local epidemiological context is not advisable.

Using antibiotics with low MICs may improve treatment efficacy; however, eradication failure can still occur due to heteroresistance, tolerance, or persistence—phenomena distinct from classic resistance. Heteroresistance involves small resistant subpopulations growing under antibiotic pressure while the majority are killed; these subpopulations are often undetectable in standard tests but may appear as colonies within inhibition zones on gradient tests. It has been reported in *S. aureus*, *Klebsiella* spp., *E. coli*, *P. aeruginosa*, and others, especially with antibiotics like colistin or fosfomycin [Band et al., 2019; Tsuji et al, 2019]. Proper dosing strategies, including loading doses and combination therapies, are recommended to counteract heteroresistance.

Tolerance describes bacterial populations surviving high antibiotic concentrations without growth, often due to genotypic mutations or phenotypic states. Such bacteria exhibit unchanged MICs but reduced bactericidal killing (MBC/MIC ratio >32) [Band et al., 2019, Li et al., 2011]. Persistence involves a small fraction of dormant cells that

survive antibiotic treatment, complicating eradication efforts [Band et al., 2019, Li et al., 2011]. These phenomena challenge therapy despite apparent *in vitro* susceptibility. MIC values are crucial for optimizing targeted antibiotic therapy when analyzed alongside pharmacokinetic parameters—volume of distribution, half-life, clearance, peak and trough concentrations, and area under the curve. These vary by patient factors and over time. In resistant Gram-negative infections, detecting resistance mechanisms (e.g., carbapenemases) informs treatment. EUCAST and CLSI agree that resistance mechanism presence does not preclude carbapenem use if MIC-based susceptibility is confirmed. For carbapenemase-producing Enterobacterales, meropenem remains a key agent at high doses and prolonged infusions, often combined with other antibiotics depending on susceptibility and MIC category (S or I). For strains with higher MICs, combination therapies and newer agents targeting specific resistance enzymes are used. Pan-resistant strains require multi-drug regimens, with MIC values guiding the choice of least resistant drugs [Fritzenwanker et al., 2018; Tsuji et al., 2019; Tumbarello et al., 2012].
Pharmacokinetic/pharmacodynamic indices (PK/PD) correlate antibiotic efficacy with MIC. Commonly, efficacy depends on:

(1) Time above MIC (T > MIC): The proportion of dosing interval during which drug levels exceed MIC. Near 100% T > MIC correlates with successful treatment, especially in immunocompromised patients and Gram-negative infections. For carbapenems and Gram-positive bacteria, lower thresholds may suffice [Sinnollareddy et al., 2012; Turnidge et al., 1998]. Lower MICs facilitate achieving T > MIC with standard dosing; higher MICs may require dose adjustments or alternative therapies. Only free (unbound) drug concentrations contribute to efficacy. Increasing drug levels far above MIC offers no additional benefit for time-dependent antibiotics [Muller et al.. 2018; Tam et al., 2020].

It becomes easier to capture the relationship between the MIC and the achievement of the PK/PD index when the interaction is presented in a graphic form and also with one of the mathematical formula for the calculation of T > MIC proposed by Turnidge in 1998 [Masich et al., 2018]:

$$\%T_{C>MIC} = \ln\left[\frac{Dose}{Vd \times MIC}\right] \times \left[\frac{t_{1/2}}{0.693}\right] \times \left[\frac{100}{DI}\right]$$

where: ln—natural logarithm, Vd—volume of distribution (L/kg), (kg), $t_{1/2}$—serum half-life (hours), elimination rate constant (h-), DI = dosing interval (hours).

(2) Cmax/MIC is a parameter characteristic for antibiotics whose effectiveness depends on maximum concentration which is many times greater than the MIC (min. 8–10×) and not on the time it is kept above the MIC [Heffernan et al., 2018]. However, as with the previously discussed parameter, lower MIC values are more likely to meet the efficacy condition for these antibiotics while reducing the risk of toxic concentrations.

(3) AUC/MIC (∫AUC/MIC): characterizes time- and concentration-dependent antibiotics [Abdul-Aziz and Roberts, 2020; Pea et al., 2005; Salem et al., 2014; Tam at al., 2018; Tsala et al., 2018; Xie et al., 2017]. As with the two previous parameters, the MIC value will influence drug effect.

Calculation of the AUC/MIC Index

The formula for calculating the AUC/MIC ratio, considering the MIC value [Mohr et al., 2004], is:

$$\frac{AUC}{MIC} = \ln\left[\frac{Dose}{Vd \times MIC}\right] \times \left[\frac{t_{1/2}}{0.693}\right] \times \left[\frac{24}{DI}\right]$$

where: ln—natural logarithm, Vd—volume of distribution (L/kg), $t_{1/2}$—serum half-life (hours), DI—dosing interval (hours).

Use of PK Parameters in Clinical Efficacy Prediction

When applying pharmacokinetic (PK) parameters to predict antibiotic efficacy, it is important to select values specific to patient groups (e.g., ICU patients, those with nosocomial pneumonia, children, pregnant women) or ideally, individualized PK data for the patient. However, individual PK measurement is challenging [Parthasarathy et

al., 2018]. Automated immunoassay analysers for serum antibiotic concentrations are available mainly for vancomycin and aminoglycosides [Parthasarathy et al., 2018], so population-based PK parameters are commonly used. Unfortunately, most available PK data are derived from healthy volunteers, whereas sick patients often exhibit altered PK, influencing treatment outcomes.

Challenges in Combining MIC and PK Data

To accurately determine PK/PD indices, MIC measurements should coincide with serum antibiotic concentrations after dosing, which is practically difficult due to rapid drug concentration changes, while MIC is a static measurement. Accelerated MIC determination would enhance therapy optimization, similar to rapid susceptibility testing directly from positive blood cultures using disk diffusion [EUCAST, 2020]. Although direct MIC methods using gradient strips have been explored [Bianco et al., 2019; Hong et al., 1996; Kontopidou et al., 2011], they have not received EUCAST or CLSI approval.

Currently, MIC90 values derived from cumulative epidemiological data may be used while awaiting individual MIC results. Statistical methods like Monte Carlo simulations estimate the probability of achieving optimal PK/PD indices based on population PK data and MIC values. These simulations help assess dosage adjustments for effective therapy [EUCAST, 2020]. Continuous updates of these models are necessary to reflect evolving clinical conditions and dosing regimens.

Limitations of MIC Testing

Antimicrobial susceptibility tests, including MIC determinations, assess direct pathogen-drug interactions in vitro and do not account for host factors such as drug distribution, protein binding, organ function, immune response, nutrition, or concurrent treatments, all of which influence clinical outcomes [Doern and Brecher, 2011; Falagas et al., 2012; Mouton et al., 2018; Puttaswamy et al., 2018]. Thus, susceptibility alone does not guarantee therapeutic success.

Higher MICs correlate with increased treatment failure risk, even when strains are categorized as susceptible. For example, vancomycin efficacy against *Staphylococcus aureus* diminishes significantly at MIC ≥ 1 mg/L [Kullar et al., 2011; Maclayton et al.,

2006; Moise-Broder et al., 2004; Sakoulas et al., 2004]. Clinical interpretation of MIC requires integration with PK parameters, demanding multidisciplinary collaboration among microbiologists, pharmacologists, and clinicians.

MIC determinations face variability; repeated measurements can differ by up to two-fold, affecting susceptibility classification and dosing decisions [Doern and Brecher, 2011]. Additionally, MIC results represent standardized inocula, not accounting for bacterial load variability at infection sites, which can influence in vivo resistance and treatment outcomes [Mouton et al., 2018; Puttaswamy et al., 2018]. Strain virulence, unreflected by MIC, also impacts therapy effectiveness [Doern and Brecher, 2011].

Timing and Practical Use of MIC Data

MIC results typically require 3–5 days, creating a gap between test ordering and availability. Meanwhile, pathogen characteristics and susceptibilities may evolve, complicating treatment decisions [Doern and Brecher, 2011]. Nonetheless, cumulative MIC data remain valuable for guiding empirical therapy and antibiotic stewardship, provided they are derived from infection isolates, not colonization or contamination, to avoid misleading conclusions.

MIC is currently the best available parameter reflecting antibiotic efficacy against bacterial strains. Despite method standardization, MIC values can vary by ± one dilution, usually without clinical impact; however, values near breakpoints warrant careful interpretation for resistance classification and optimal therapy selection.

Incorporating MIC with individualized PK parameters can greatly improve treatment outcomes. While direct PK/PD parameter measurement is complex and often inaccessible, Monte Carlo simulations provide useful estimates linking MIC and dosing strategies. EUCAST's introduction of two susceptibility categories facilitates tailored dosing based on MIC.

Laboratory determination of MIC remains challenging and time-consuming. Even precise MIC values cannot always predict clinical success due to undetectable mechanisms like heteroresistance, tolerance, or persistence, which contribute to treatment failures despite optimal antibiotic choice.

MIC values are crucial for optimizing targeted antibiotic therapy when analyzed alongside pharmacokinetic and pharmacodynamic parameters. **Pharmacokinetics (PK)** refers to how the body absorbs, distributes, metabolizes, and eliminates the antibiotic, which influences drug concentrations at the site of infection. Important PK parameters include the volume of distribution (Vd), serum half-life (t1/2), clearance, peak and trough concentrations, and area under the concentration-time curve (AUC). These parameters vary among patients depending on factors such as age, weight, organ function, and disease state.

Pharmacodynamics (PD), on the other hand, studies how the antibiotic affects the bacteria—its mechanism of action and the relationship between drug concentration and bacterial killing or growth inhibition. Combining PK and PD (referred to as PK/PD) helps determine the optimal dosing regimen to maximize antibiotic effectiveness while minimizing toxicity and resistance development.

By integrating MIC values with individual PK and PD parameters, clinicians can better tailor antibiotic therapy to achieve the most effective bacterial eradication.

Comparison of Kirby-Bauer and MIC Methods (Optional)

Both Kirby-Bauer disk diffusion and Minimum Inhibitory Concentration (MIC) methods are essential tools in antimicrobial susceptibility testing, each with its advantages and limitations.

Advantages of Kirby-Bauer:

- Simple, cost-effective, and widely accessible.
- Requires minimal specialized equipment and technical expertise.
- Suitable for rapid screening of bacterial susceptibility in routine clinical laboratories.

Disadvantages of Kirby-Bauer:

- Provides only qualitative results (Susceptible, Intermediate, Resistant).
- Less precise for antibiotics with narrow therapeutic windows or where dosing optimization is critical.
- Results can be influenced by variations in agar depth, inoculum size, and incubation conditions.

Advantages of MIC:

- Provides quantitative measurement of antibiotic activity, giving the lowest concentration inhibiting bacterial growth.
- Enables precise dosing adjustments, especially in complicated or resistant infections.
- Supports integration with pharmacokinetic/pharmacodynamic (PK/PD) data for optimized therapy.

Disadvantages of MIC:

- More labour-intensive and time-consuming.
- Requires specialized equipment and trained personnel.
- Generally higher cost compared to disk diffusion.

When to Use Each Method:

Kirby-Bauer is preferred for routine, large-scale susceptibility screening due to its simplicity and cost-effectiveness. MIC testing is recommended when precise quantitative data is needed for clinical decision-making, such as in severe infections, treatment failures, or infections caused by multidrug-resistant organisms.

Discussion / Practical Examples

Interpretation of antimicrobial susceptibility tests must consider both laboratory methodology and clinical context. Some practical considerations include:

- A bacterial isolate may be categorized as susceptible by Kirby-Bauer but have an MIC near the resistance breakpoint, necessitating careful clinical interpretation.
- Variability in inoculum density, agar composition, or incubation time can cause inconsistent disk diffusion results. Strict adherence to standardized protocols is essential.
- Resistance mechanisms like heteroresistance or bacterial persistence may lead to treatment failure despite in vitro susceptibility.
- Errors such as disk contamination, improper storage, or incorrect inoculum preparation can invalidate test results.

Real-world examples demonstrate that correlating laboratory data with patient outcomes improves therapy success and informs antibiotic stewardship.

Conclusion

Accurate antimicrobial susceptibility testing is a cornerstone of effective infection management and antimicrobial stewardship. Understanding the strengths and limitations of Kirby-Bauer and MIC methods ensures appropriate test selection and result interpretation. Ongoing quality control, method standardization, and interdisciplinary collaboration are vital to optimize antibiotic use and combat resistance.

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CHAPTER 3. THE TOXIC EFFECTS OF CONTAMINANTS ON THE HUMAN BODY AND INNOVATIVE GREEN METHODS TO REDUCE THEM

3.1 Introduction

The sustainable production of natural products, through careful selection of plant resources and preservation of their active principles, offers promising pathways to mitigate the toxic effects of environmental contaminants on the human body, forming a cornerstone of innovative green strategies for pollution reduction.

Across the globe, environmental toxicants and chemical contaminants have emerged as a serious and expanding public health challenge. They originate from a wide range of human activities — including industrial manufacturing, intensive farming, fossil fuel combustion, and poor waste management — and are now present in air, soil, water, and even the food supply. The World Health Organization (WHO 2023) reported that in 2016, nearly a quarter of all deaths and disease burdens worldwide were linked to environmental factors that could be prevented, such as chemical pollution and hazardous waste.

The consequences of these pollutants for human health range from short-term effects like respiratory distress and skin irritation to chronic and life-threatening conditions. Air pollution alone is believed to claim over five million lives each year (Augusta University, 2023). Certain chemicals can act as carcinogens, teratogens, or mutagens, causing permanent biological harm.

Exposure occurs through multiple routes — breathing contaminated air, consuming polluted food and water, or skin contact (Sokan-Adeaga et al., 2023). Once absorbed, these substances can damage vital organs such as the lungs, heart, liver, kidneys, brain, and reproductive organs, with children, the elderly, and pregnant women being the most vulnerable (Balbus et al., 2013).

The problem is not only medical but also economic. Lead poisoning alone is estimated to cost the global economy approximately US $6 trillion annually — about 6.9% of the

world's GDP (WHO 2023). In 2019, children under five were estimated to have lost 765 million IQ points collectively due to lead exposure (World Bank, 2023), with lasting consequences for human development and productivity.

Climate change is expected to make matters worse by altering patterns of pollutant release, distribution, and human vulnerability (Balbus et al., 2013). These shifts could change risk profiles, making it essential to re-evaluate current strategies for assessing and managing environmental health hazards.

This chapter examines how environmental contaminants affect the human body and presents innovative green approaches aimed at reducing their impact, supporting the global vision of moving toward a pollution-free future (UNEP, 2023).

3.2 Toxic effects on human body

Chemical pollution

The use of chemicals has increased dramatically in some sectors, particularly industry, agriculture, and transportation. Since 1950, more than 140,000 new chemicals and pesticides have been synthesized; over 3,000 of these are used in very large quantities (over 300,000 kg per year), leaking into the environment, and all living things are exposed to them.

Human exposure occurs through the air we breathe, the water we drink or wash with, the food we eat, the soil we touch, the utensils we use, detergents, cosmetics, etc.

We are exposed practically everywhere: at home, at school, at work, in parks, in the countryside, at the seaside and in the mountains, and while traveling. Less than 45% of these substances have been studied for basic toxicity, and less than 10% for effects on children.

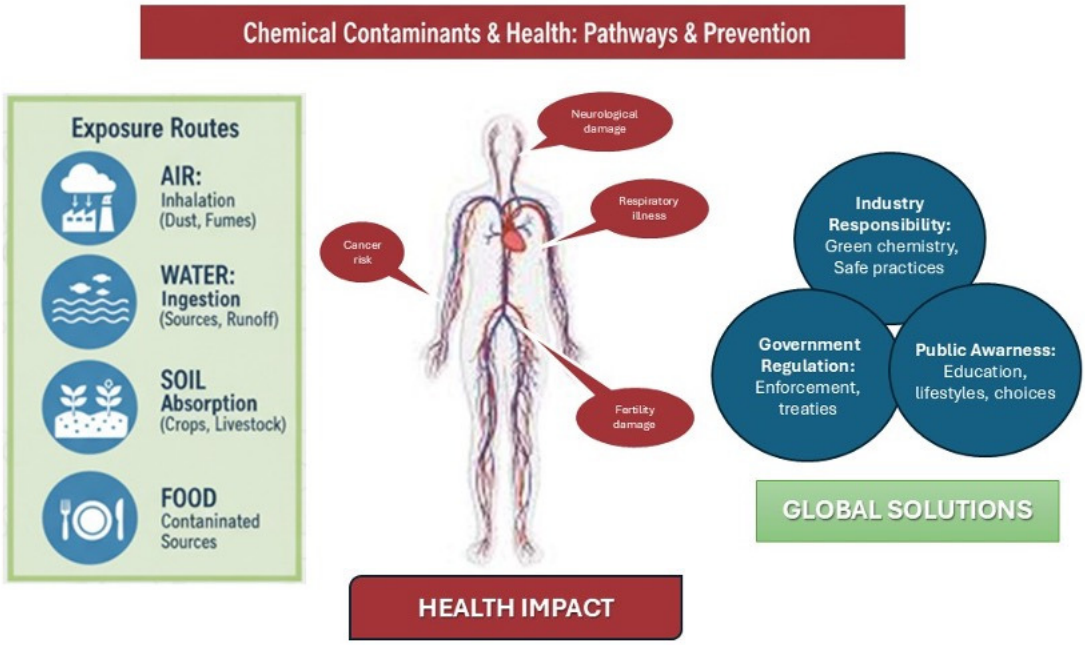

Figure 3.1 Chemical Pollutants and Health: Exposure Pathways and Prevention Strategies at the Global Level

According to the World Health Organization, the 10 greatest chemical hazards to public health are: lead, asbestos, mercury, arsenic, benzene, dioxins, highly toxic pesticides, cadmium, fluoride, and substances fixed in particulate matter in general. Chemicals can cause adverse health effects, often due to long-term exposure.

Dioxins and dioxin-like substances, including polychlorinated biphenyls (PCBs), are considered persistent organic pollutants (POPs), meaning they persist in the environment, accumulate in living organisms, and can be harmful to health. They are often found in remote regions of the world because they are transported by water, air, and other means over long distances from their source. Chemicals that act as endocrine disruptors are particularly dangerous to health.

Drug pollution

Drugs, though highly useful and sometimes essential for survival, have become a cause for concern worldwide as "emerging" environmental contaminants (contaminants that until now had not caused concern, i.e., one could speak of an

"emerging concern"). This is because their increasingly widespread use has led to their dispersion into the environment, as residues can spread during production, use (eliminated in urine, feces, sweat), and disposal.

Residues of various types of drugs (hormones, anticancer drugs, antidepressants, antibiotics, etc.) have been found in surface water, groundwater, drinking water, soil, air, and wildlife worldwide. The quantities of individual drugs are minimal, but the molecules (i.e., the active ingredients contained in the various drugs) present are numerous, their environmental distribution is global, and animals and people are exposed to many mixtures of these substances for long periods.

All this raises concerns that even at minimal concentrations—levels of nanograms (a billionth of a gram) or micrograms (a millionth of a gram) per liter—drugs and their residues could pose a risk to human health, especially since significant adverse effects on animals are already known. It's not easy to pinpoint the primary source of pollution.

Most of the medications we consume are eliminated through urine, feces, or sweat, and thus end up in wastewater (wastewater). Many medications are applied as creams, lotions, and medicated patches, and the portion that isn't absorbed by the skin is eliminated during showers or baths, again ending up in wastewater. A portion of the unused and expired medications that fill the typical medicine cabinet in every Italian home are not disposed of properly. Hospitals and private nursing homes can also be a source of pollution; in fact, they are facilities where many medications are used, and wastewater purification systems are often inadequate to filter these substances. Pharmaceutical companies are a significant source of pollution, although not all to the same extent.

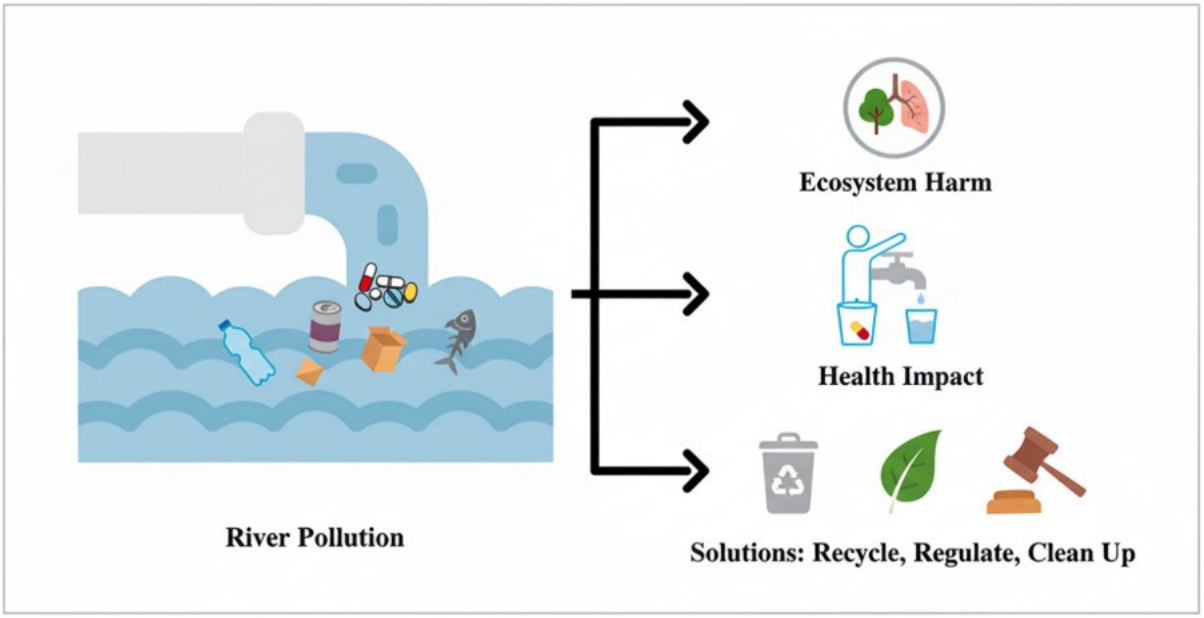

Figure 3.2. Pharmaceutical pollution and sustainable development goals

Drugs are designed to be biologically active (i.e., to affect the body's cells) even at low concentrations, to last a long time, and are often non-biodegradable (i.e., they do not degrade quickly in the environment). Therefore, they tend to persist in the environment and accumulate (bioaccumulate) in biota, that is, the diverse animal or plant organisms that live in an ecosystem. Recent research has shown that drugs accumulate in aquatic invertebrates, which are then ingested by fish, which in turn can be eaten by humans.

This phenomenon, known as "biomagnification," contaminates the food chain. It is not easy to study the effects of minimal amounts of drugs on the human body. Chronic effects (due to long-term exposure) could be due to minor alterations that are not easy to identify in time.

When, after many years, the damage becomes evident, it is not always possible to determine whether there is a correlation between exposure to the drug and the disorder from which a person suffers. The cumulative effect of even minimal quantities of pharmaceutical products in drinking water is worrying, particularly among the most vulnerable population groups (children, pregnant women, people with disabilities, etc.).

The most feared aspect, whose effects are already evident, is indirect environmental exposure to antibiotics, which can create antibiotic-resistant bacteria and thus expose humans to the risk of infections from untreatable bacteria. Despite the uncertainty about the effects on human health, there is considerable evidence that pharmaceuticals in water affect aquatic life and beyond! Many studies report clear and significant effects due to drug contamination of water and the environment, such as the feminization of fish and the sterility of frogs caused by residues from the contraceptive pill. Studies of fish upstream and downstream of wastewater treatment plants have found more female and intersex fish downstream. In Pakistan, diclofenac (a powerful anti-inflammatory) has caused the deaths of many thousands of vultures that feed on carcasses contaminated with the drug. Diclofenac, at the concentrations found in freshwater, also causes lesions in the kidneys and gills of trout. Sulfadiazine, an antimicrobial used in pig farming, causes antibiotic resistance in soil bacteria.

Plastic pollution

The release of plastic-derived particles—microplastics (0.1 to 5,000 micrometers (µm)) and nanoplastics (0.001 to 0.1 µm, i.e., 1 to 100 nanometers)—into the environment is a global problem.

Microplastics and nanoplastics are widespread throughout marine and terrestrial ecosystems.

Human exposure can occur through the ingestion of fish, shellfish, oysters, mussels, contaminated water, salt, or even through inhalation of contaminated air. Of concern are the high concentrations of hazardous and endocrine-disrupting substances that can be ingested through microplastics, such as bisphenol A (from packaging), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs).

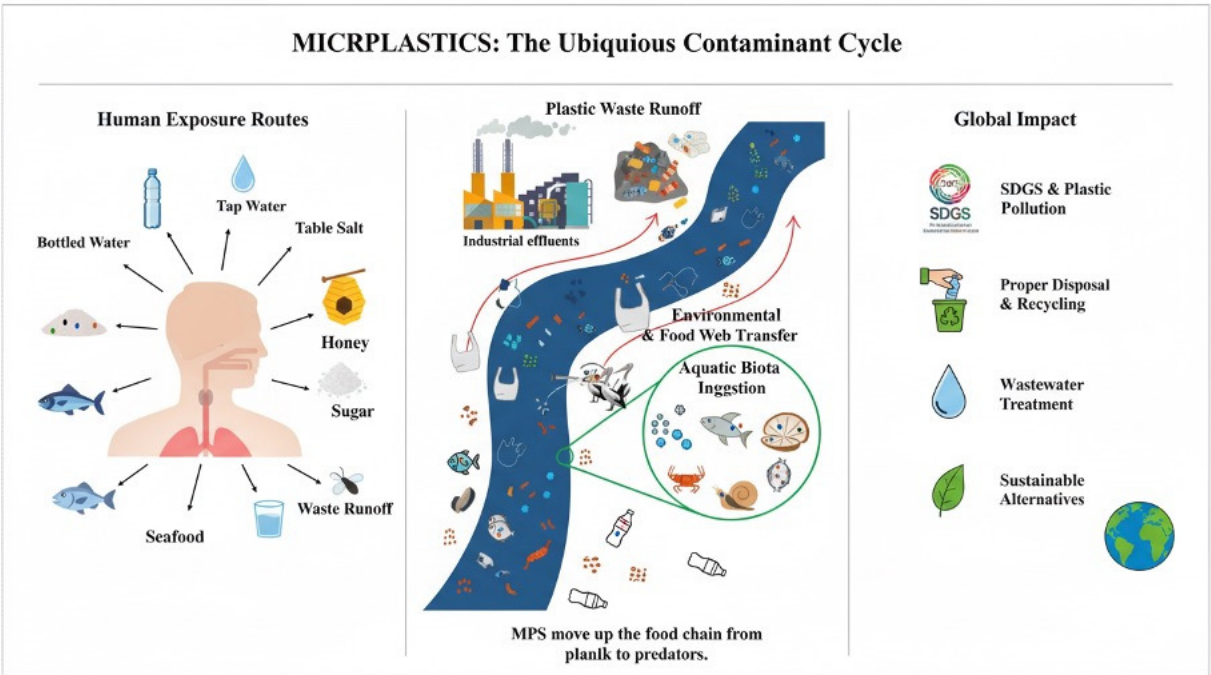

Figure 3.3 Micro(nano)plastics pollution and human health

Others

The influence of environmental pollutants on human health is broad and complex, forming the central focus of environmental toxicology—a discipline dedicated to understanding how chemicals, whether acting alone or in combination, may harm living organisms over time (Shetty, 2023). Even at low concentrations, many substances can interact in ways that amplify their toxic potential, making long-term exposure particularly hazardous.

General Toxicological Impacts

Pollutants have been linked to a wide spectrum of illnesses, including cancer, ischemic heart disease, chronic obstructive pulmonary disease (COPD), stroke, neurological and mental disorders, and diabetes (Shetty, 2023). Some contaminants accumulate selectively in certain organs, reaching internal concentrations that exceed those found in the surrounding environment. This bioaccumulation process can, over years, cause significant and sometimes irreversible organ damage (Alharbi, 2018).

Notable examples of harmful substances include:

- DDT – a pesticide historically used against agricultural pests and still applied in some regions of Africa, Asia, and Latin America.
- Furans – compounds arising from high-temperature cooking, certain chemical processes, and some consumer products such as packaging materials.
- Dioxins – toxic by-products of industrial processes or natural events like forest fires and volcanic eruptions.
- Volatile organic compounds (VOCs) – easily evaporating chemicals found in paints, solvents, fuels, building materials, and cleaning agents.
- Aldehydes – for instance, formaldehyde, used in pressed wood products, textiles, and cosmetics.
- Volatile heavy metals – such as mercury vapor from industrial activities, coal burning, or waste decomposition.
- Chlorinated hydrocarbons – present in some solvents and cleaning products.
- Drugs.

In certain cases, substances are harmless in their original form but become toxic after metabolic conversion inside the body. These resulting metabolites can be even more damaging, sometimes possessing carcinogenic properties.

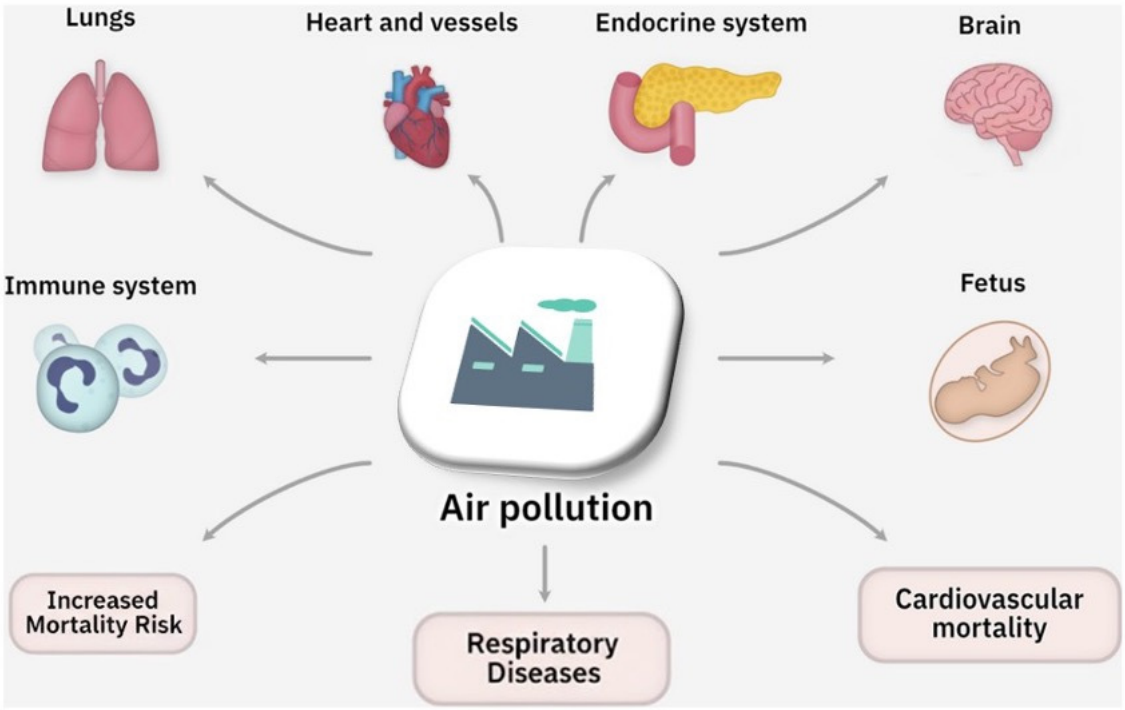

Figure 3.4 Adverse effects of air pollution on human health

Impacts on Specific Organ Systems

Respiratory System

Airborne pollutants—such as carbon monoxide, ozone (O_3), nitrogen dioxide (NO_2), sulfur dioxide (SO_2), particulate matter, and heavy metals—can lead to both acute and chronic respiratory illnesses, including bronchitis, pneumonia, COPD, and asthma (Shetty, 2023). Prolonged exposure may cause permanent structural damage to the lungs, hinder lung growth in children, and elevate lung cancer risk (Schraufnagel, 2019).

The environment is a source of potentially harmful exposures to human health. It is estimated that approximately 7% of the annual disease burden in Europe is associated with environmental risk factors. In particular, numerous studies have highlighted the harmful role of air pollution, documenting a broad spectrum of health effects. With an estimated 7 million premature deaths, air pollution is considered the leading

environmental cause of disease and death worldwide. The term "air pollution" refers to a mixture of gases and particles contained in the air we breathe. Air pollution is generated by both human activities (motor vehicle emissions, industrial processes, building heating) and natural events (forest fires, volcanic eruptions, dust storms). Air pollutants can be transported for thousands of kilometers, affecting air quality even in places distant from their source. However, pollution levels are generally higher in areas close to the emission source. It is estimated that today more than 90% of people living in urban areas are exposed to levels of air pollutants higher than those indicated by the World Health Organization (WHO) Guidelines.

Air pollutants can be classified as primary or secondary, depending on how they form. Primary pollutants are emitted directly by human-induced processes, such as carbon monoxide emitted from a motor vehicle's exhaust or sulfur dioxide emitted from factories. Secondary pollutants are formed when primary pollutants react in the atmosphere. A very important secondary pollutant is ozone, which arises from chemical reactions between primary pollutants and sunlight.

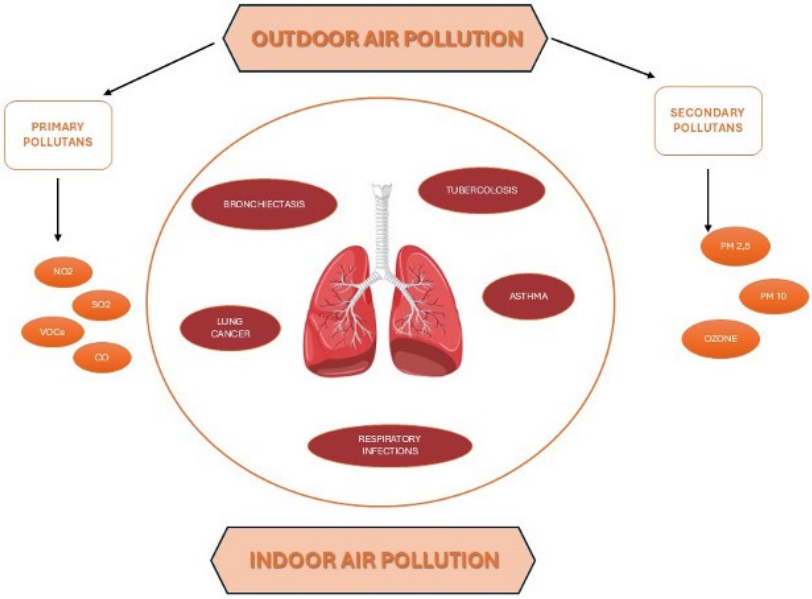

Figure 3.5 Air pollution exposure—the (in) visible risk factor for respiratory diseases

In addition to outdoor pollution, indoor pollution also represents a significant health risk. Indoor air quality is affected by both external and internal sources of pollution. External sources derive from outdoor pollutants that typically enter through open windows, while internal sources can arise from combustion processes or include building materials, furniture, and commonly used household cleaning products. The indoor environment therefore contributes significantly to exposure to pollutants, many of which are higher in concentration indoors than outdoors.

Cardiovascular System

Several studies have shown that air pollution not only affects the lungs but can also damage the heart. Now, a new international study goes a step further, showing how not only air, but also soil and water pollution are closely linked to cardiovascular disease (Münzel, 2025). Fine particulate matter ($PM_{2.5}$) and similar pollutants can constrict blood vessels, weaken cardiac muscle, and promote inflammatory processes that contribute to atherosclerosis, hypertension, and heart attacks (Dai, 2024). Globally, air pollution is estimated to be responsible for 19% of cardiovascular deaths and 21% of stroke fatalities (Schraufnagel, 2019).

Pollution from heavy metals, pesticides, and micro- and nanoplastics can damage the cardiovascular system, causing oxidative stress, inflammation, and disrupting circadian rhythms.

Exposure to toxic chemicals, such as heavy metals, solvents, dioxins, and pesticides, can occur in the workplace, through common household products, or through environmental contamination, contributing to blood vessel dysfunction and the development of cardiovascular disease. Soil contamination is a much less visible threat to human health than dirty air, but growing evidence demonstrates that pollutants in soil and water can damage cardiovascular health. This occurs through some central mechanisms that have been identified as key factors in the atherosclerotic process, such as inflammation of the vascular system, increased oxidative stress.

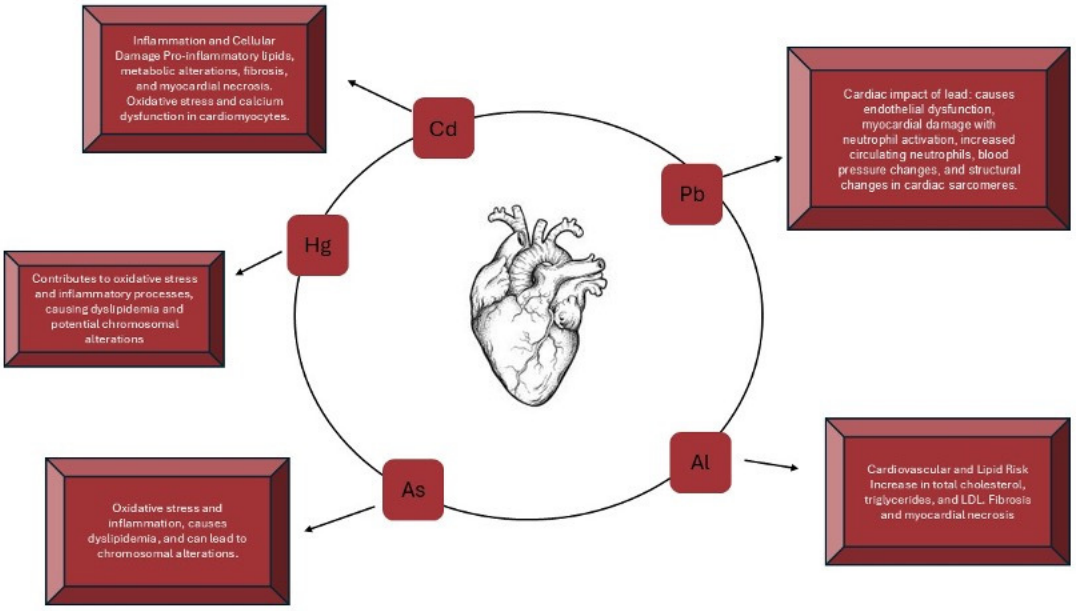

Figure 3.6 Impact of the environmental pollution on cardiovascular diseases

Nervous System

The nervous system is vulnerable to pollution and environmental exposure to harmful substances, known as neurotoxins. The effects of these molecules can affect the functioning and integrity of nervous tissue to the point of contributing to the development of certain nervous system disorders.

It is estimated that approximately 30% of all synthetic chemicals have neurotoxic properties, and unfortunately, most of the substances used or released into the environment do not have solid evidence of their safety.

Environmental neurotoxins are numerous, including, for example, pyrethroid and organochloride pesticides, as well as nanoplastics.

Pollutants in the air are associated with reduced cognitive performance in children and heightened risks of dementia and stroke in older adults (Dai, 2024). These effects are believed to result from oxidative stress and inflammation in neural tissue, which may accelerate neurodegenerative processes.

Exposure and subsequent excessive metal accumulation in the body can cause toxicological repercussions. In particular, accidental acute events or, more frequently, environmental or occupational exposure can negatively impact the nervous system. The metals most commonly implicated are mercury, lead, arsenic, cadmium, and manganese.

The main sources of exposure include contaminated water or food, occupational exposure (e.g., steelworks), dental amalgam, smoking, metal alloys, pigments, batteries, insecticides, and hair dyes.

Several studies highlight a possible association of organophosphates with certain neurodegenerative diseases. These substances can interfere with neuronal function and the action of neurotransmitters, as well as promote oxidative stress and neuroinflammation, which is inflammation of the nervous system. Furthermore, some research suggests that exposure to organophosphates may increase the risk of autoimmune responses in some people, potentially affecting the structure and function of nerve transmission.

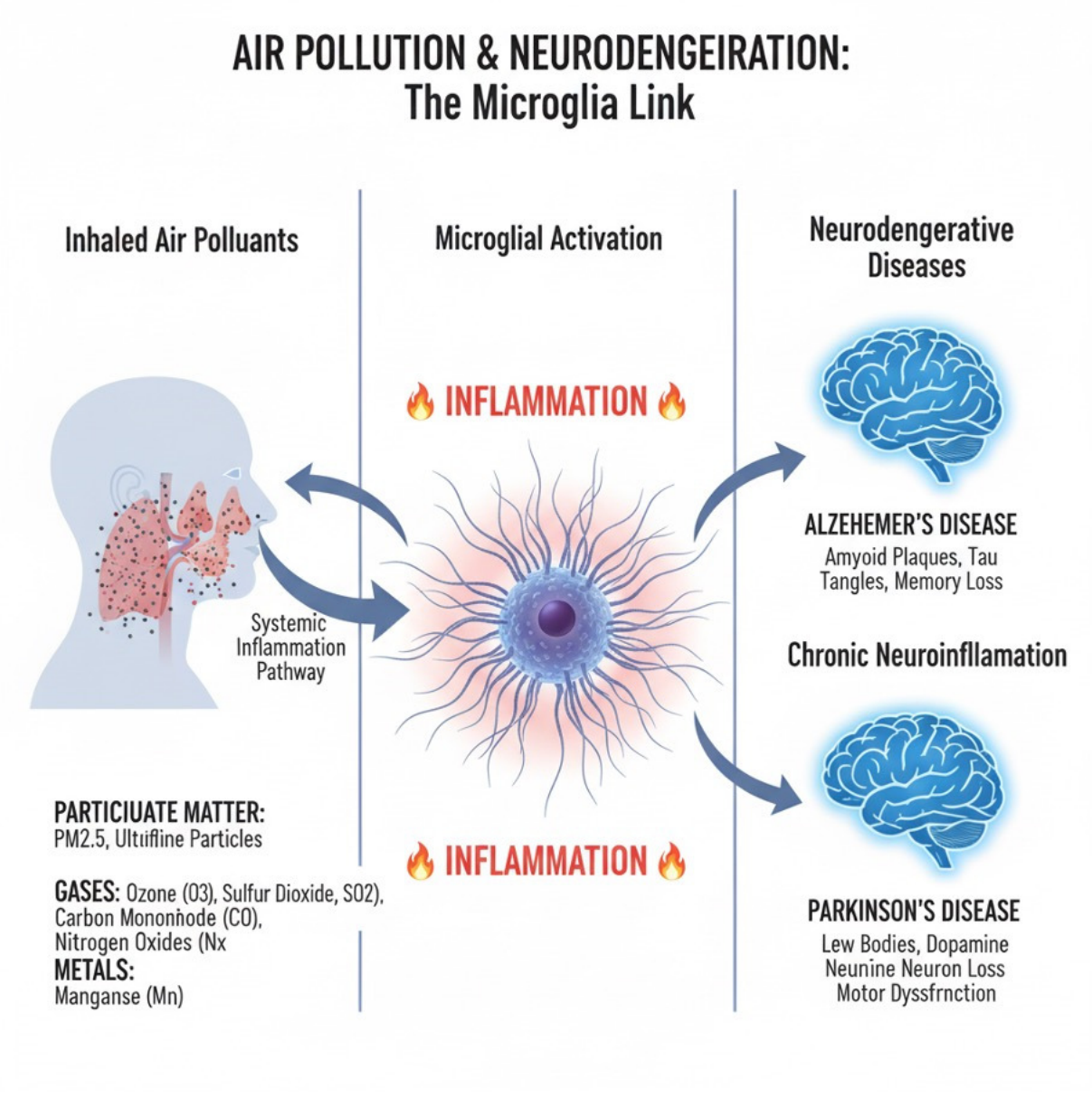

Figure 3.7 Air pollution increases neuroinflammation, especially microglial activation, which may be a key mechanism involved in air pollution-induced AD and PD

Endocrine System

A specific group of contaminants known as endocrine-disrupting chemicals (EDCs) can interfere with hormone production, metabolism, and receptor binding (Kumar, 2020). Exposure has been associated with reproductive and developmental issues, immune system changes, and an increased risk of hormone-sensitive cancers.

Endocrine disruptors (EDs) include a wide range of chemicals that can alter the hormonal balance of living organisms, including humans. EDs interfere with the normal biochemical signals released by our body's endocrine glands, which regulate extremely delicate functions: the immune system, the function of certain endocrine glands (e.g., the thyroid), metabolism, reproductive functions, and neuropsychiatric functions.

Pathologies induced by frequent exposure to minimal doses of EDs include: thyroid and neurodevelopmental disorders (cognitive, behavioral, and relational disorders), increased miscarriage rates, reduced fertility, genital and reproductive abnormalities, endometriosis, obesity and type 2 diabetes, tumors, and immune-mediated diseases. EDs act insidiously, even at minimal doses, particularly during crucial developmental stages, such as intrauterine life or early childhood. Exposure to EDs can also cause alterations to gametes (sperm and eggs), resulting in health risks that could be passed down through generations. Endocrine-disrupting chemicals include dioxins, PCBs (polychlorinated biphenyls), and various pesticides, as well as substances found in our everyday environment and consumer products, such as flame retardants, phthalates, and bisphenol A.

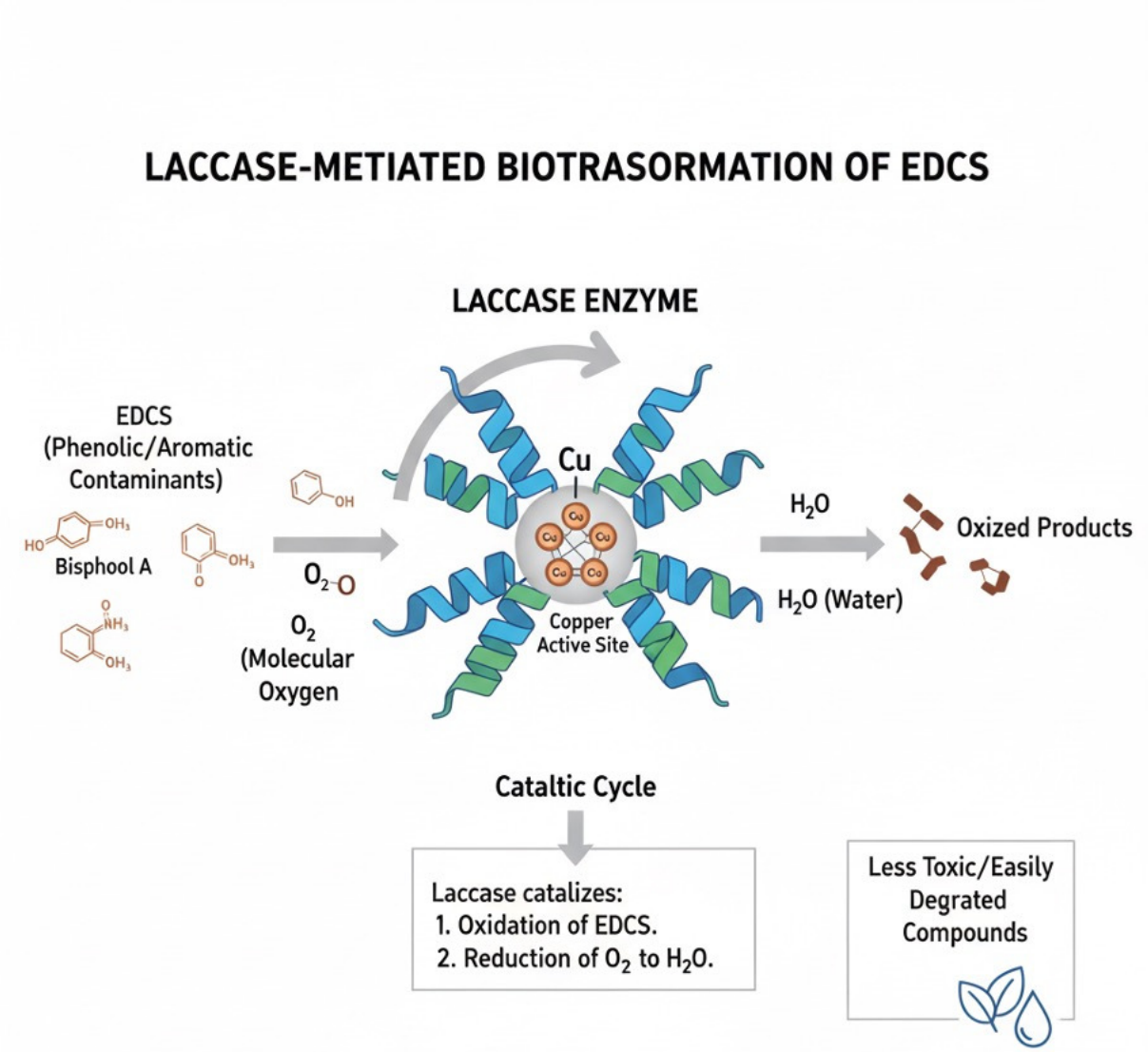

Figure 3.8 Endocrine disrupting chemicals (EDCs) in environmental matrices

Chronic Diseases and Disorders

Respiratory diseases: Long-term pollution exposure is a major risk factor for COPD and asthma and is linked to increased mortality from these conditions.

Metabolic disorders: Pollutants can induce oxidative stress and inflammation, leading to insulin resistance and a greater likelihood of type 2 diabetes (Dai, 2024).

Neurological disorders: Continuous exposure is connected to Alzheimer's disease, dementia, and other degenerative brain conditions through inflammatory and oxidative mechanisms.

Cancer: Fine particulate matter ($PM_{2.5}$) is recognized as a carcinogen, contributing to lung, bladder, and certain pediatric cancers (Schraufnagel, 2019).

Endocrine disruption: EDCs can alter puberty timing, impair fertility, reduce semen quality, and increase risks of hormone-sensitive malignancies (Kumar, 2020).

In summary, environmental contaminants affect human health through multiple pathways, often targeting more than one organ system at a time. The interplay between various pollutants can intensify their harmful effects, underlining the urgent need for integrated environmental health policies and pollution control strategies.

3.3 Pathways of Exposure

Understanding how environmental contaminants enter the human body is essential for accurately assessing and mitigating associated health risks. This section outlines the primary mechanisms through which environmental toxicants are released, transported, and ultimately reach human populations.

Environmental Release and Transport

Contaminants may be introduced into the environment through industrial processes, agricultural activities, waste disposal, and other anthropogenic actions. Once released, they can move through different environmental compartments:

- Air - Pollutants may be directly emitted into the atmosphere or volatilize from soil and water. Air currents can transport these substances over long distances (United States Environmental Protection Agency, 2024a).
- Water - Contaminants can enter rivers, lakes, and oceans via direct discharge, surface runoff, or leaching into groundwater. Water bodies not only act as transport systems but also serve as points of human exposure (United States Environmental Protection Agency, 2024b).

- Soil and Sediment - Pollutants may settle and accumulate in soils through atmospheric deposition or irrigation with contaminated water. Soil can act as a long-term reservoir, later releasing contaminants back into air or water (Alharbi et al., 2018).

Routes of Human Exposure

Humans can be exposed to environmental toxicants via three primary routes:

- Inhalation - Breathing in contaminated air that contains gases, vapours, aerosols, or particulate matter. Indoor air can also be affected when outdoor pollutants infiltrate buildings (United States Environmental Protection Agency, 2024c).
- Ingestion - Swallowing contaminants present in food, drinking water, or soil and dust particles. This includes accidental soil ingestion in children and hand-to-mouth transfer of residues (United States Environmental Protection Agency, 2024b; New Hampshire Department of Environmental Services, 2024).
- Dermal Contact - Direct skin contact with contaminated soil, water, or consumer products containing hazardous chemicals. Occupational exposure is a frequent contributor to dermal uptake (New Hampshire Department of Environmental Services, 2024).

Factors Influencing Exposure

The extent and severity of exposure depend on several factors:

- Duration - Short-term (acute) vs. long-term (chronic) exposure affects health outcomes differently.
- Intensity - Higher concentrations of contaminants increase potential risk.
- Frequency - Repeated exposure can compound health effects.
- Individual Susceptibility - Certain groups, including pregnant women, children, the elderly, and immunocompromised individuals, are more vulnerable (New Hampshire Department of Environmental Services, 2024).

Bioaccumulation and Biomagnification

Persistent organic pollutants and heavy metals can build up in body tissues over time through bioaccumulation. When these contaminants pass along the food chain, they may become more concentrated at higher trophic levels-a process known as biomagnification (United States Environmental Protection Agency, 2024b).

Exposure Assessment

Evaluating human exposure involves:

- Identifying contamination sources
- Tracing environmental transport and fate
- Determining exposure points and routes
- Defining at-risk populations

These assessments allow classification of exposure pathways as completed, potential, or eliminated, providing the basis for effective risk management strategies (Agency for Toxic Substances and Disease Registry, 2024).

3.4 Evaluation of contaminants

Climate change, environmental pollution, biodiversity loss, and the unsustainable use of natural resources collectively pose significant risks to human, animal, and ecosystem health. These threats include infectious and non-communicable diseases, antimicrobial resistance, and water scarcity. Ensuring a healthy planet for all requires more effective monitoring, reporting, prevention, and remediation of pollution affecting air, water, soil, and commodities (European Commission, 2021).

The scale of the pollution problem can be greatly reduced through strong governmental action, advanced infrastructure, and the application of modern technologies. However, achieving the goal of a clean environment is hindered by several challenges, including insufficient public engagement in pollution control initiatives and inadequacies in ecological management systems.

Addressing pollution effectively requires the use of the latest technological solutions and targeted research to better understand the mechanisms by which contaminants accumulate in the environment.

Food contamination remains a critical concern, as elevated chemical concentrations in edible products pose serious health hazards. Contaminants in food may be naturally occurring in the environment or introduced through human activities. Furthermore, contamination can occur at multiple points along the food chain - during production, processing, packaging, transportation, and storage (Rather et al., 2017).

Food safety challenges can be grouped into four key categories:

- Microbiological safety
- Chemical safety
- Personal hygiene
- Environmental hygiene

With the globalization of the food trade, food has become a major route of human exposure to pathogenic microorganisms responsible for foodborne illnesses, potentially entering at various stages of the value chain. Tracking and detecting these pathogens - particularly bacteria - back to their sources remains a challenge for producers, processors, distributors, and consumers alike.

Food safety and nutrition are closely interlinked. Unsafe food can trigger a vicious cycle of disease and malnutrition, disproportionately affecting infants, young children, the elderly, and individuals with compromised health. Because food supply chains now span multiple national and regional borders, ensuring food safety in the 21st century will require strong collaboration between governments, producers, suppliers, distributors, and consumers (Fung et al., 2018).

In recent years, various analytical methods have been developed for detecting contaminants in different matrices. Since contaminants are often present at extremely low concentrations, a very low detection limit is required, and sample preparation becomes essential to reduce matrix effects in food analysis. Sample preparation may

involve multiple steps - such as filtration, pH adjustment, extraction, clean-up, and enrichment - to ensure analytes are detected at suitable concentration levels.

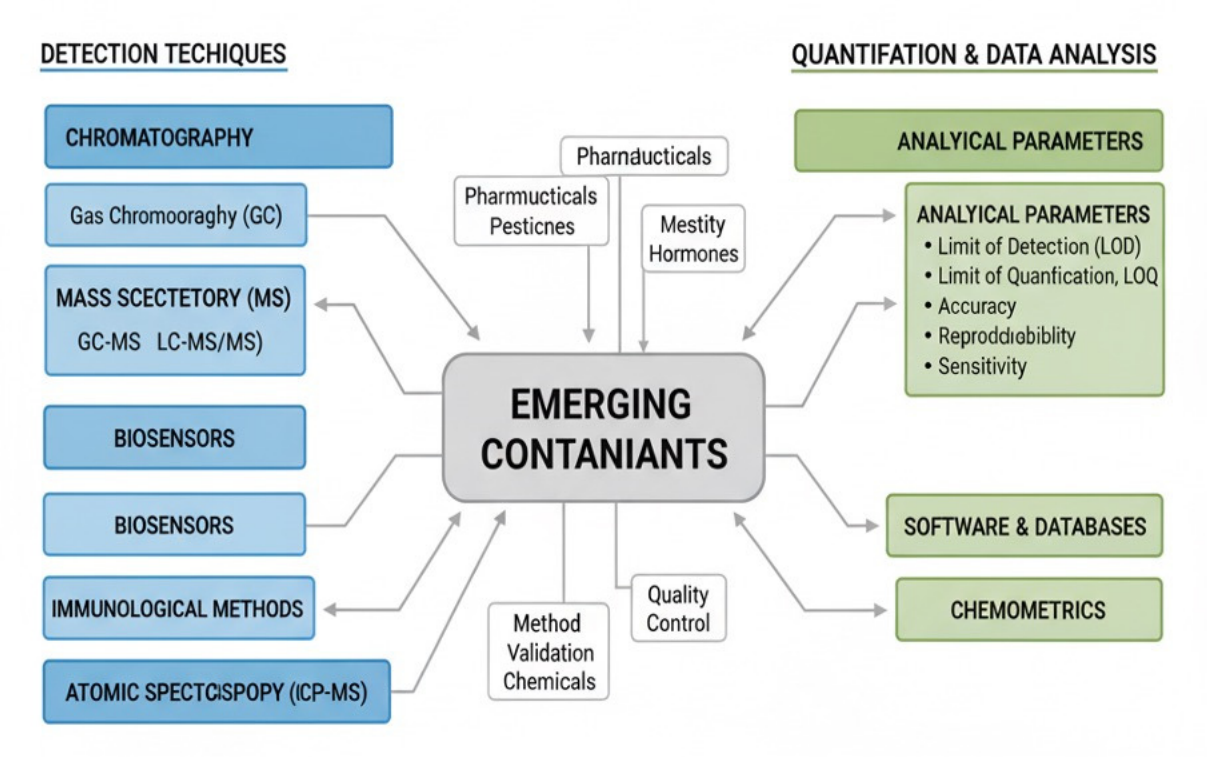

Figure 3.9 Techniques for the detection and quantification of emerging contaminants

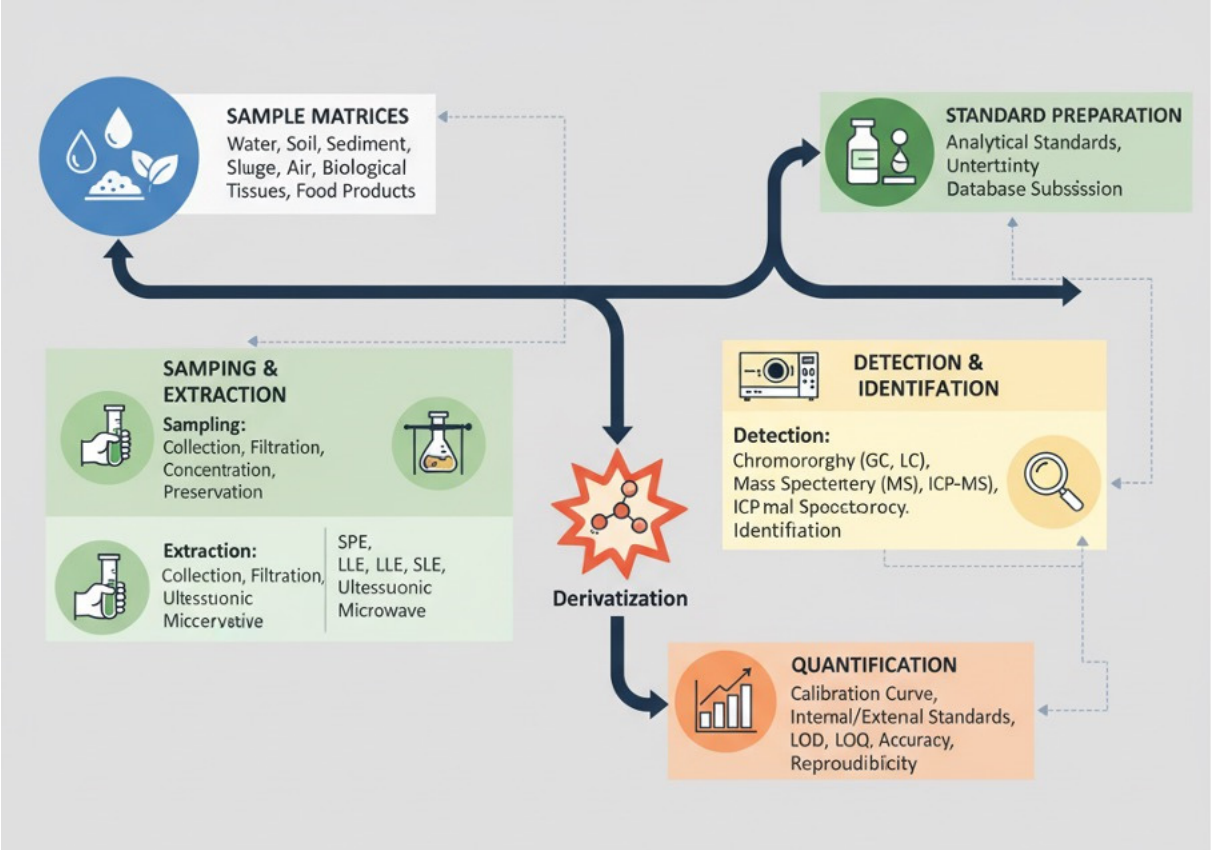

Figure 3.10 General workflow for the analysis of contaminants

A wide range of sample preparation techniques is now available, including supercritical fluid extraction, solid-phase extraction, solid-phase microextraction, microwave-assisted extraction, liquid-liquid extraction, liquid-phase microextraction, pressurized liquid extraction, and stir bar sorptive extraction (Guo et al., 2019).

3.5 Innovative green methods to reduce the toxic effects of contaminants

Green chemistry, also known as sustainable chemistry, is a modern approach in the chemical sciences that has evolved significantly since the 1990s. It is defined as “the use of chemical techniques and methodologies that reduce or eliminate the use or generation of raw materials, products, by-products, solvents, and reagents that are hazardous to human health or the environment” (United States Environmental

Protection Agency). At its core, this philosophy is based on sustainability, encapsulated in twelve fundamental principles:

- Prevention – Avoid generating waste rather than treating or disposing of it after creation.
- Atom economy – Design synthesis methods to incorporate as much of the starting materials as possible into the final product.
- Less hazardous synthesis – Employ processes that use and generate substances with minimal toxicity.
- Designing safer chemicals – Preserve chemical functionality while minimizing toxicity.
- Safer solvents and auxiliaries – Avoid auxiliary substances (e.g., solvents) where possible or make them non-hazardous when required.
- Energy efficiency – Minimize energy demands; perform reactions at ambient temperature and pressure when feasible.
- Renewable feedstocks – Use renewable rather than depletable raw materials when technically and economically viable.
- Reduce derivatives – Avoid unnecessary derivatization steps that require extra reagents and generate waste.
- Catalysis – Use catalytic reagents, which are more efficient than stoichiometric ones.
- Design for degradation – Ensure that products break down into harmless substances at the end of their lifecycle.
- Real-time analysis – Develop monitoring techniques to detect and prevent hazardous substances during processing.
- Inherently safer chemistry – Select substances and process forms that reduce the risk of accidents such as explosions or releases.

Sustainable Alternatives to Conventional Pollutant Removal

The growing presence of pollutants — including heavy metals, organic compounds, pharmaceuticals, and emerging contaminants — poses significant environmental and public health risks. Traditional removal methods such as chemical precipitation, ion

exchange, and membrane filtration often face limitations, including high costs, high energy demand, and the generation of secondary pollutants.

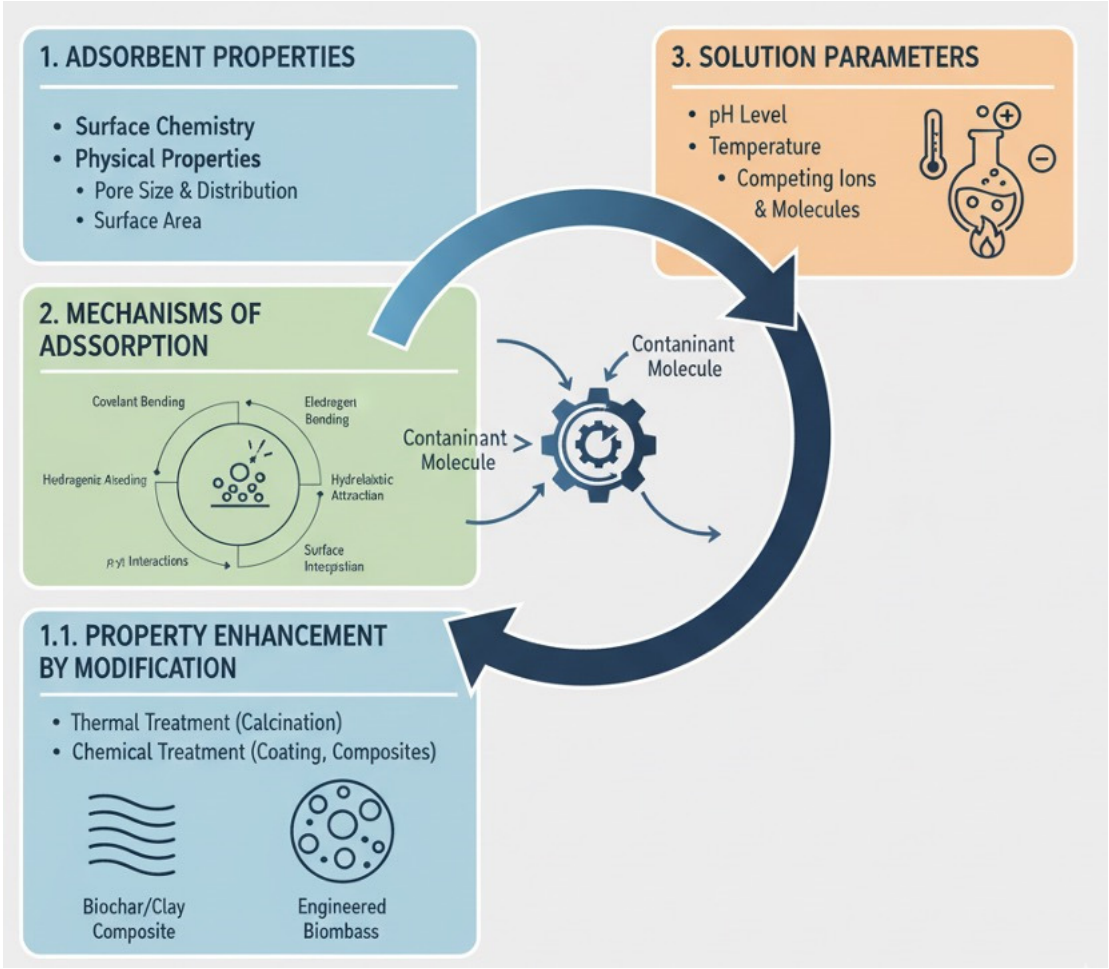

Figure 3.11 Factors that influence the adsorption capacity of low-cost adsorbents

Recent research highlights the potential of non-conventional adsorbents as more sustainable alternatives. Materials such as nanocellulose, chitosan-based nanocomposites, and metal–organic frameworks (MOFs) have shown superior performance in terms of adsorption capacity, selectivity, and reusability, making them attractive for environmental applications (Akhtar et al., 2024).

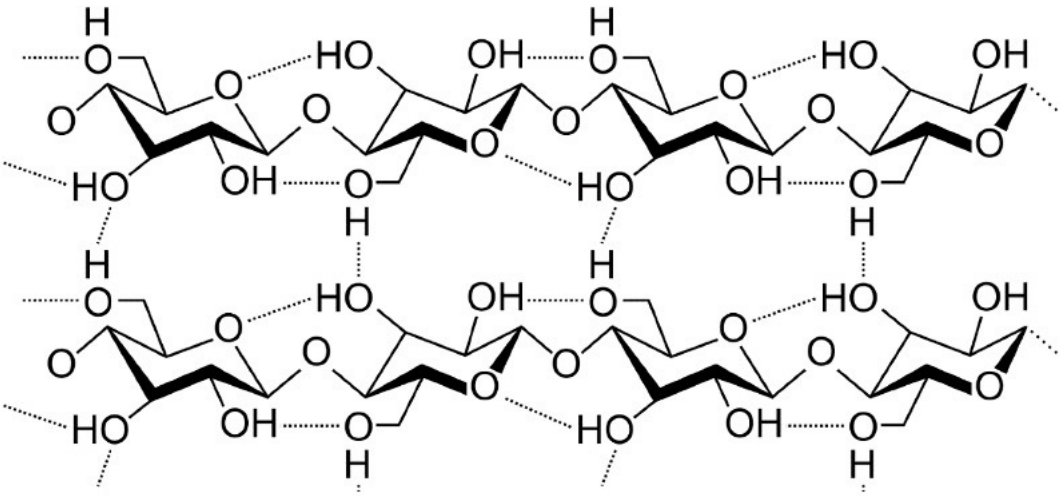

Figure 3.12 Chemical structure of cellulose and their hydrogen bonds

Green Chemistry Solutions

1. Safer solvents and reaction conditions

Replacing hazardous solvents with safer alternatives is a major innovation in green chemistry. For example, water-based paints have replaced solvent-based coatings, eliminating toxic fumes and reducing air pollution without compromising performance. Running chemical reactions at ambient temperature and pressure further reduces energy consumption and minimizes hazards.

Water-based paints have largely replaced solvent-based coatings in many applications due to environmental and health concerns. Government regulations focused on reducing volatile organic compounds (VOCs) have driven this shift, as water-based paints emit significantly fewer VOCs. While solvent-based paints were once the standard, water-based alternatives are now favoured for their lower toxicity, quicker drying times, and reduced environmental impact.

2. Renewable feedstocks

Green chemistry prioritizes feedstocks derived from renewable resources, such as agricultural by-products, over fossil fuels or mined materials. This reduces environmental impact, conserves non-renewable resources, and often results in more

biodegradable products. A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable. Nature produces about 170 billion tons of plant biomass annually; of which we currently use about 3.5 percent for human needs. It is estimated that about 40 billion tons of biomass, or about 25 percent of the annual production, would be required to completely generate a bio-based economy. The technical challenge in the use of such renewable feedstocks is to develop low energy, non-toxic pathways to convert the biomass to useful chemicals in a manner that does not generate more carbon than is being removed from “thin air”

3. Catalysis and atom economy

Catalysts enable reactions to occur efficiently with minimal waste, often replacing stoichiometric reagents that are used in excess. At the same time, designing reactions for high atom economy ensures that the majority of input materials are incorporated into the final product. A primary goal of green chemistry is the minimization or preferably the elimination of waste in the manufacture of chemicals and allied products. This necessitates a paradigm shift in the concept of efficiency in organic synthesis, from one that is focused on chemical yield to one that assigns value to minimization of waste. What is the cause of waste? The key lies in the concept of atom economy: “synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product”.

4. Designing for degradation

Products are increasingly designed to degrade into harmless substances after use, reducing persistence in the environment and lowering hazardous waste management costs. Green chemistry practitioners aspire to optimize the commercial function of a chemical while minimizing its hazard and risk. Trade-offs, or alternative approaches, must be evaluated when the molecular features to be designed in for commercial function overlap with those to be designed out to reduce hazard and risk.

Biological Remediation of Heavy Metals

Rapid industrialization has intensified heavy metal contamination of soils worldwide, posing serious ecological and health threats. Removal and neutralization of these contaminants is now a global priority. Bioremediation — using microorganisms such

as bacteria, microalgae, yeast, and fungi — is gaining attention as an eco-friendly and cost-effective alternative, particularly effective at low metal concentrations (Maqsood et al., 2022).

Often, integrated methods combining physicochemical and biological processes are used to achieve optimal results across the heavy metal treatment cycle. These approaches restore contaminated environments into healthier, life-supporting systems while minimizing environmental side effects.

Green Toxicology

An emerging discipline called Green Toxicology provides a framework for integrating the principles of toxicology into the enterprise of designing safer chemicals, thereby minimizing potential toxicity as early in production as possible. Green toxicology merges the principles of green chemistry with toxicology to ensure chemical safety from the earliest stages of product design (Maertens et al., 2024). It employs modern, non-animal testing strategies, including in silico computational models, artificial intelligence predictions, and human cell-based assays, allowing for faster and more cost-effective hazard assessments compared to traditional animal testing.

Figure 3.12 Green Toxicology: Connecting Green Chemistry and Modern Toxicology

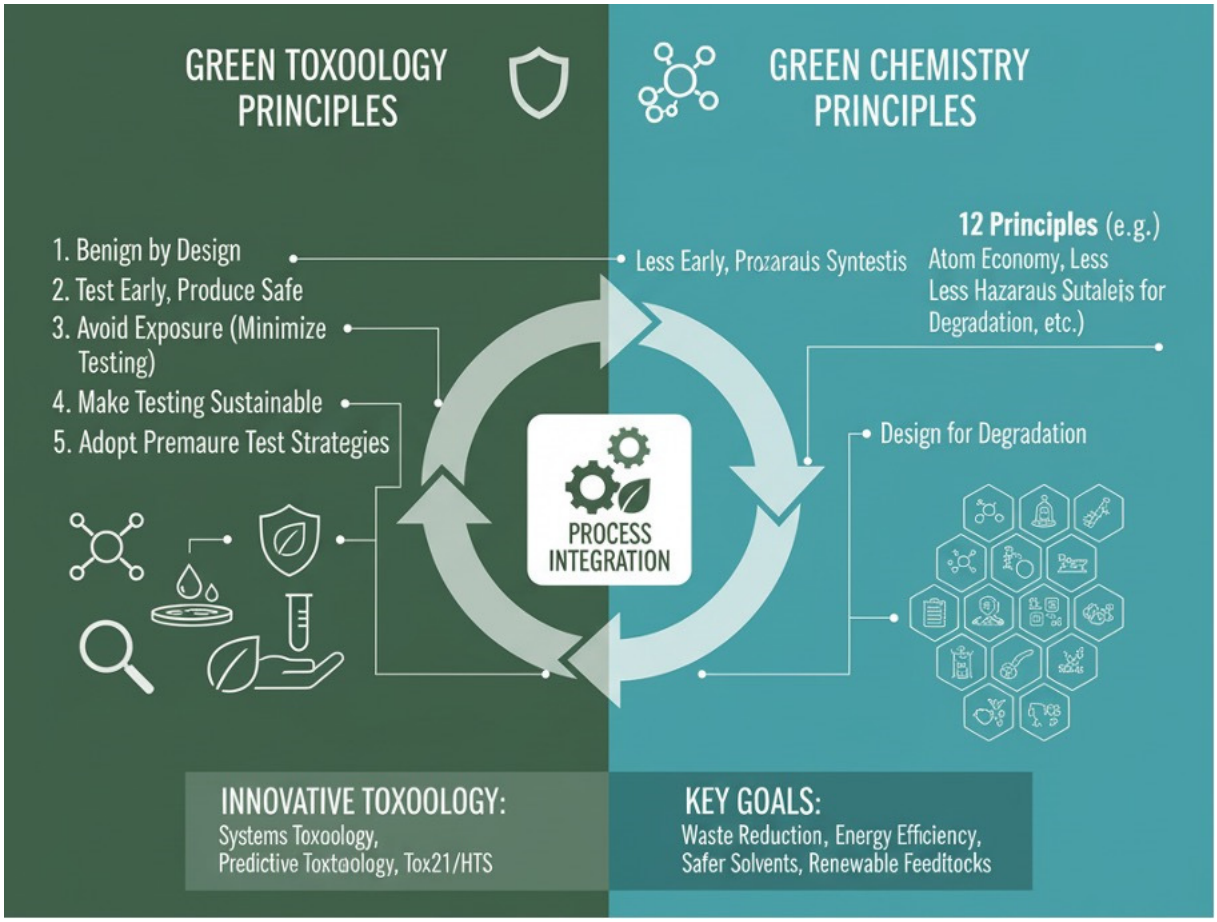

Figure 3.13 Principles of green toxicology and green chemistry and how they integrate into the chemical production process

Key elements of green toxicology include:

- Applying alternative, validated test methods.
- Incorporating safety considerations early in chemical design.
- Assessing life cycle impacts across supply chains.
- Prioritizing prevention over remediation.
- Benefits of Green Chemistry and Toxicology
- Human Health
- Cleaner air and water through reduced hazardous emissions.
- Improved workplace safety in chemical industries.

- Safer consumer products and food.
- Environmental
- Reduced greenhouse gas emissions, smog formation, and ozone depletion.
- Minimized ecological disruption from chemical pollution.
- Lower need for hazardous waste disposal.
- Economic
- Higher reaction yields and lower raw material costs.
- Reduced waste disposal expenses.
- Increased plant efficiency and energy savings.
- Competitive advantage through eco-friendly product labeling.

Challenges and Future Directions

While green chemistry and toxicology offer clear benefits, adoption faces barriers such as validation of new methods, regulatory acceptance, and institutional resistance to change. Future priorities should include:

- Expanding the portfolio of validated alternative test methods.
- Embedding green chemistry concepts into education and industrial practice.
- Creating policy incentives for sustainable innovation.
- Strengthening collaboration between chemists, toxicologists, and environmental scientists.

Innovative green methods represent a transformative pathway for reducing the toxic effects of contaminants on both human health and the environment. By integrating the principles of green chemistry, embracing biological remediation strategies, and applying green toxicology frameworks, we can design and implement safer, more sustainable chemical processes. This approach supports a long-term vision of a cleaner, healthier, and more resilient planet.

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CHAPTER 4. THE ROLE OF HEALTHY NUTRITION AND APPROVED USE OF SAFE FOOD SUPPLEMENTS: INNOVATIVE APPROACHES AND AWARENESS EVALUATION

4.1 Introduction

Nutrition is a cornerstone of human health, shaping growth, immune competence, cognitive performance, and lifelong risk for infectious and non-communicable diseases. Yet the world now faces a “double burden” of malnutrition: undernutrition persists while overweight and obesity have risen sharply—patterns documented in the Global Nutrition Report 2020 (Global Nutrition Report, 2020). Improving dietary quality through whole foods—vegetables, fruits, whole grains, legumes, nuts, seeds, and healthful oils—remains the most effective population strategy, as reflected in major dietary guidance frameworks (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2020).

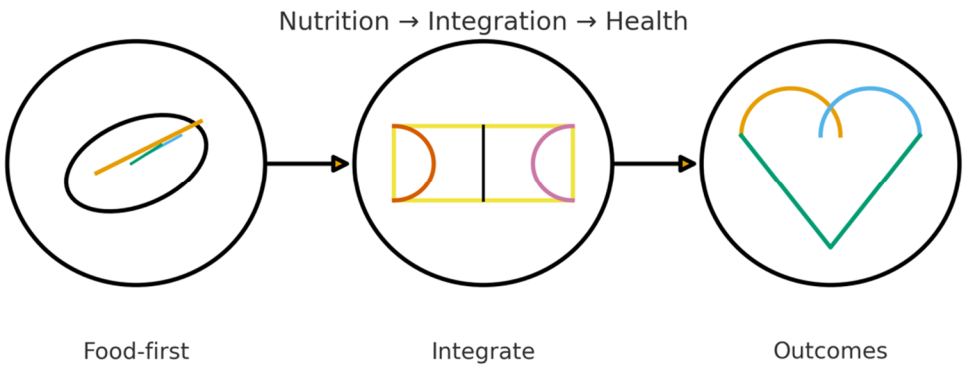

Figure 4.1 Schematic representation of nutrition integration in human health

Diet quality and disease prevention

Robust prospective cohorts and randomized trials link plant-forward, fiber-rich patterns to lower cardiometabolic risk. A comprehensive Lancet systematic review and meta-analysis found higher dietary fiber intakes associated with 15–30% relative risk reductions in all-cause and cardiovascular mortality and in type 2 diabetes incidence; benefits rose up to roughly 25–29 g/day of fiber (Reynolds et al, 2019). In parallel, the PREDIMED randomized trial reanalyzed in 2018 showed that a Mediterranean diet

enriched with extra-virgin olive oil or nuts reduced major cardiovascular events versus a lower-fat control diet (Estruch et al, 2018). These data support prioritizing minimally processed plant foods rich in fibers and polyphenols as a sustainable, high-value foundation for health promotion.

Leveraging plant bioactives and bioavailability

Plants supply vitamins, minerals, fibers, and thousands of bioactive compounds (for example, polyphenols, carotenoids, glucosinolates). Their health value depends not only on content but also on bioavailability—how well compounds are released, absorbed, and reach targets. Simple food-based strategies can meaningfully enhance uptake. For example, adding vitamin C–rich foods to meals with plant (non-heme) iron increases iron absorption, as demonstrated in classic human balance studies (Hallberg et al, 1989). Such synergies illustrate how optimizing food combinations and preparations can amplify the benefits of plant-derived actives.

Dietary supplements: roles, limits, and regulation

Dietary supplements (vitamins, minerals, botanicals, probiotics, and others) can help correct specific, documented deficiencies or meet higher needs at certain life stages, but they do not replace a high-quality diet (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2020). Regulatory frameworks differ: in the United States, the Dietary Supplement Health and Education Act (DSHEA, 1994) classifies supplements as a category of foods; manufacturers are responsible for safety and labeling, and products generally enter the market without pre-market FDA approval (U.S. Congress, 1994). In the European Union, Directive 2002/46/EC harmonizes rules for food supplements, including permitted vitamins and minerals and labeling requirements (European Parliament and Council, 2002). At the same time, post-market investigations have repeatedly found adulteration of some supplements with undeclared pharmaceuticals, underscoring the need for vigilance and for using verified products from reputable manufacturers (Tucker et al, 2018).

When supplements are clearly beneficial

Folic acid 400 µg/day for all who could become pregnant, starting before conception through the first trimester, reduces neural tube defects and is strongly recommended

by health authorities (U.S. Preventive Services Task Force, 2023). Iodine sufficiency is essential for thyroid function and neurodevelopment; iron and vitamin B12 require targeted use when deficiency or malabsorption is present, guided by clinical evaluation.

Situations where “more” is not better

For primary prevention in generally healthy adults, large randomized trials show no overall reduction in major cardiovascular events or cancer with routine vitamin D supplementation (Manson et al, 2019). Supplement decisions should be individualized, integrating measured deficiency, clinical context, and risk–benefit considerations, not solely label claims.

Practical, sustainable priorities

1. Food-first: Aim for at least 25–30 g/day of dietary fiber from whole grains, legumes, vegetables, fruits, nuts, and seeds; this level aligns with observed risk reductions in the Lancet meta-analysis (Reynolds et al, 2019).
2. Plant-forward patterns: Emphasize Mediterranean-style meals rich in extra-virgin olive oil, nuts, legumes, whole grains, and abundant produce, as supported by PREDIMED (Estruch et al, 2018).
3. Targeted supplementation: Use evidence-based supplements for defined needs, with particular attention to periconceptional folic acid (U.S. Preventive Services Task Force, 2023).
4. Beware of claims: Be cautious with products promising disease treatment or rapid body composition change; adulteration has been documented in weight-loss, sexual-enhancement, and bodybuilding categories (Tucker et al, 2018).
5. Policy and systems: The double burden of malnutrition calls for food environments and policies that make nutrient-dense foods accessible and affordable (Global Nutrition Report, 2020).

4.2 Fundamentals of Healthy Nutrition

The optimal capitalization of plant resources—through careful selection of species, preservation of bioactive compounds, and use of effective forms of preparation and intake—underpins a dietary pattern that supports long-term health. Emphasizing minimally processed plant foods rich in fiber, polyphenols, carotenoids, and unsaturated fats aligns individual choices with population guidance and sustainability goals (WHO, 2020; Lichtenstein et al., 2021).

Figure 4.2 Balanced plant-forward plate: Key elements of healthy nutrition, showing nutrient-rich, minimally processed foods, quality fats, and evidence-based dietary targets.

Essential nutrients and their roles

A healthy diet provides six fundamental nutrient groups—proteins, carbohydrates, fats, vitamins, minerals, and water—each with distinct physiological functions in tissue maintenance, energy metabolism, cellular structure, fluid–electrolyte balance, and

enzymatic regulation. Quality within these groups matters: complex carbohydrates and dietary fiber support glycemic control and cardiometabolic health; replacing saturated fats with unsaturated fats improves lipid profiles; and adequate micronutrient intake sustains immune, neuromuscular, and skeletal function (WHO, 2020; Hooper et al., 2020; Reynolds et al., 2019).

Principles of a balanced, plant-forward pattern

Dietary balance emphasizes variety, nutrient density, and energy intake matched to need while limiting highly processed foods. Widely endorsed targets include consuming at least 400 g/day of fruits and vegetables, keeping free sugars below 10% of total energy, and prioritizing fat quality by limiting saturated fat and eliminating industrial trans fats in favor of unsaturated oils. Replacing refined grains with whole grains, and diversifying protein sources with legumes, nuts, seeds, and fish, further enhances overall diet quality (WHO, 2020; Aune et al., 2016; Lichtenstein et al., 2021).

Nutrient density and fiber quality

Nutrient-dense foods deliver more vitamins, minerals, and bioactive compounds per calorie. Higher intakes of dietary fiber from whole grains, legumes, fruits, and vegetables are associated with 15–30% lower all-cause mortality and reduced incidence of coronary heart disease, stroke, type 2 diabetes, and colorectal cancer in prospective cohorts; randomized trials show lower body weight, systolic blood pressure, and total cholesterol at higher fiber intakes. Pragmatic targets of at least 25–29 g/day are supported by pooled evidence and confer additional benefits for gut function and inflammation (Reynolds et al., 2019).

Fat quality and cardiometabolic risk

Shifting from saturated to polyunsaturated fats lowers cardiovascular risk. Emphasizing oil-rich plant foods (nuts, seeds, olive and other vegetable oils) and fish, while limiting high-SFA foods and eliminating industrial trans fats, improves LDL-cholesterol and downstream clinical endpoints, consistent with global and specialty-society prevention guidance (Hooper et al., 2020; Lichtenstein et al., 2021).

Dietary patterns with clinical outcome data

Food-based patterns that operationalize these principles demonstrate event-level benefits. In a large Spanish primary-prevention trial, a Mediterranean diet supplemented with extra-virgin olive oil or nuts reduced major cardiovascular events compared with a control diet, underscoring the value of quality-focused dietary patterns beyond macronutrient counting (Estruch et al., 2018; Lichtenstein et al., 2021).

Cancer prevention considerations

Authoritative syntheses recommend maintaining healthy body weight; emphasizing whole grains, vegetables, fruit, and pulses; limiting ultra-processed foods, red and processed meats, and alcohol; and avoiding excess sodium and free sugars to lower the risk of several cancers. These strategies dovetail with cardiometabolic guidance, illustrating cross-disease benefits of a minimally processed, plant-forward pattern (WCRF/AICR, 2018).

Food processing and eating context

Beyond nutrient totals, processing level and food matrix affect appetite and energy intake. In a tightly controlled crossover trial, ad libitum ultra-processed diets led to higher energy intake and weight gain within two weeks versus minimally processed diets matched for offered calories, macronutrients, sugar, sodium, and fiber, suggesting texture, palatability, and eating rate can drive passive overconsumption (Hall et al., 2019).

Implementation, equity, and life-course perspectives

Adoption depends on affordability and access, culinary skills, cultural preferences, and the food environment. Practical, scalable steps include building meals around vegetables, whole grains, and legumes; choosing unsalted nuts and seeds for snacks; using oils rich in unsaturated fats; planning weekly fish; and reserving refined grains, sweets, and processed meats for occasional use. Public-health measures that improve access to healthy foods and temper the marketing and availability of ultra-processed products are central to closing nutrition equity gaps across the life course (WHO, 2020; Lichtenstein et al., 2021).

4.3 Food Supplements: Regulation, Safety, and Scientific Evaluation

Food supplements, also called dietary supplements, are concentrated sources of nutrients or other bioactive substances intended to complement the usual diet when habitual intake is inadequate. Their use has expanded worldwide, supported by consumer interest in preventive health and the growth of products containing vitamins, minerals, botanicals, probiotics, omega-3 fatty acids, and other compounds. Despite widespread availability, questions about quality, safety, and clinical effectiveness remain central to scientific evaluation and regulatory oversight (NIH ODS, 2023).

Figure 4.3 Visual summary of key elements in food supplement regulation, safety, and scientific evaluation.

Regulatory framework

In the United States, dietary supplements are regulated as a category of foods under the Dietary Supplement Health and Education Act (DSHEA, 1994). Manufacturers are responsible for ensuring safety and proper labeling before marketing; the Food and Drug Administration (FDA) generally acts post-market to address adulterated or misbranded products, and it reviews notifications for “new dietary ingredients” with supporting safety data at least 75 days before marketing (FDA, 2023). Facilities must comply with current Good Manufacturing Practice requirements in 21 CFR Part 111,

which govern identity testing, purity, strength, composition, and recordkeeping, but these rules do not constitute pre-market approval and lapses in compliance can still lead to variable quality (FDA, 2007; FDA, 2023). Other jurisdictions regulate supplements as foods with specific compositional and labeling rules and typically restrict disease claims to those authorized by evidence reviews; structure/function claims must not be misleading and require a disclaimer that the product is not intended to diagnose, treat, cure, or prevent disease (FDA, 2023).

Safety considerations

Most supplements are well tolerated when used as directed, yet several risk domains require attention. Quality defects—such as substitution, contamination, or variable potency—have been documented, particularly in some botanical products; DNA barcoding surveys have identified cases of adulteration or filler use that would not be apparent to consumers (Newmaster et al., 2013). Excess intake above tolerable upper intake levels can produce toxicity; for example, high-dose vitamin E in the SELECT trial was associated with an increased risk of prostate cancer in men (Klein et al., 2011). Pharmacokinetic and pharmacodynamic interactions are well described: St John's wort induces CYP3A4 and P-glycoprotein, reducing exposure to many medicines; vitamin K antagonizes warfarin; calcium, iron, and magnesium can impair absorption of certain antibiotics and thyroid hormone. Special populations—including pregnant or lactating individuals, children, older adults with polypharmacy, and patients with renal or hepatic disease—require individualized risk assessment (NIH ODS, 2023). Post-market safety relies on adverse-event reporting by firms and clinicians to FDA systems, with enforcement actions when serious risks are identified (FDA, 2023).

Scientific evaluation of efficacy

Claims for supplements range from correcting defined deficiencies to supporting general health or reducing disease risk. Robust evaluation follows principles used in other areas of clinical science: biologic plausibility, product characterization and standardization, pharmacokinetics and bioavailability, randomized controlled trials (RCTs) with hard clinical endpoints, and systematic evidence synthesis. Large RCTs illustrate the need for precise questions and appropriate outcomes. In the VITAL trial,

vitamin D3 (2000 IU/day) and marine omega-3 fatty acids (1 g/day EPA+DHA) did not reduce the primary composite of invasive cancer plus major cardiovascular events in generally healthy adults, although secondary analyses suggested modest reductions in myocardial infarction for omega-3s and in cancer mortality after excluding early follow-up, highlighting the nuance of subgroup and endpoint interpretation (Manson et al., 2019). By contrast, pooled evidence supports certain uses: probiotics reduce the risk of antibiotic-associated diarrhea in many settings, though effects depend on strain, dose, and population (Goldenberg et al., 2017). These examples show why product-specific evidence, standardization, and transparent reporting are essential for translating mechanistic promise into reliable clinical recommendations.

Quality assurance and informed choice

Because legal compliance does not guarantee product performance, independent quality programs can help reduce uncertainty. Third-party verification (for example, the United States Pharmacopeia Verified Mark) evaluates manufacturing practices, ingredient identity and potency, specified contaminants, and label accuracy, offering an additional layer of assurance to clinicians and consumers selecting products for at-risk patients or for research use (USP, 2021). Best practice in clinical care is to prioritize food-first strategies; confirm a specific indication for supplementation (documented deficiency, increased physiological need, limited intake, malabsorption, or evidence-based therapeutic use); review drug–supplement interactions; select verified products with appropriate doses that respect tolerable upper intake levels; and monitor outcomes and adverse effects (NIH ODS, 2023; FDA, 2023).

Policy and research priorities

Strengthening pre- and post-market oversight, improving transparency of new dietary ingredient notifications, expanding analytical surveillance for adulterants, and facilitating pragmatic trials of well-characterized products would close evidence gaps. For botanicals, standardized extracts with defined chemotypes and attention to bioavailability enhancers are needed to ensure reproducibility across studies. For microbiome-targeted products, strain-resolved labeling, viable counts through shelf life, and endpoint-specific RCTs will improve interpretability (Goldenberg et al., 2017).

Alignment among regulators, third-party certifiers, clinicians, researchers, and industry can advance a marketplace in which safe, high-quality, evidence-based supplements complement—not replace—nutritionally adequate diets (FDA, 2023; NIH ODS, 2023).

4.4 Innovative Approaches to Nutrition Education

Addressing the global challenges of malnutrition and unhealthy dietary patterns requires education models that move beyond didactic delivery toward approaches that are theory-informed, participatory, and adaptable to diverse cultural and socioeconomic contexts. Contemporary programmes increasingly combine behaviour-change science, hands-on learning, community partnership, and digital tools to improve diet quality and sustain change over time (Michie et al., 2011; Bandura, 2004).

Figure 4.4 Core elements of modern nutrition education: habits, games, cooking, and community

Theory-informed design and behaviour change techniques

Effective nutrition education is anchored in established behaviour-change frameworks that diagnose what must shift for healthier habits—capability, opportunity, and

motivation—and then align techniques (goal setting, self-monitoring, feedback, problem-solving) to those targets (Michie et al., 2011). Social Cognitive Theory further emphasises self-efficacy, observational learning, and outcome expectancies; interventions that build skills (e.g., label reading, meal planning), provide credible role modelling, and reinforce successes tend to yield stronger and more durable effects (Bandura, 2004). Practically, this translates into curricula that sequence knowledge with practice (e.g., shop-and-cook sessions) and embed prompts for reflection, planning, and social support.

Gamification and interactive tools

Serious games and gamified challenges can make complex nutrition concepts tangible and intrinsically motivating, particularly for children, adolescents, and young adults. Points, levels, feedback, and narrative quests transform label reading, portion estimation, and meal composition into experiential learning. Meta-analytic evidence indicates that digital games for health behaviours can improve nutrition knowledge and self-reported intakes, especially when grounded in behavioural theory and combined with real-world practice tasks (e.g., photo-logging, cooking missions) (DeSmet et al., 2014). To maximise impact, designers should specify the targeted behaviours (e.g., ≥5 portions/day fruit and vegetables), link mechanics to those behaviours (e.g., points for verified servings), and provide timely, personalised feedback.

Hands-on and experiential learning

Culinary skills training, school gardens, tasting sessions, and sensory-based workshops translate advice into ability. Programmes that teach mise-en-place, knife skills, budgeting, and time-saving techniques reduce practical barriers to home meal preparation and are associated with more frequent cooking, higher fruit-vegetable intake, and improved diet quality in adults and families (Reicks et al., 2014). Sensory education approaches (e.g., guided exploration of taste, texture, aroma) help children accept a wider variety of foods and can increase willingness to try unfamiliar vegetables and legumes (Reverdy et al., 2008). Embedding these activities in routine settings—classrooms, community centres, primary care—supports reach and maintenance.

Community-centred and culturally responsive approaches

Community-driven nutrition education leverages local leaders, foodways, and social networks to ensure relevance and equity. Co-design with participants, incorporation of culturally preferred foods, and peer-led delivery can strengthen trust and adoption, while addressing structural barriers (affordability, access, time) through complementary strategies such as produce vouchers, mobile markets, or default "healthy choice" changes in canteens. Foundational texts emphasise that tailoring content and delivery to community norms, literacy levels, and food environments is central to effectiveness and sustainability (Contento, 2016).

Technology-enhanced learning and just-in-time support

Mobile apps, text-message coaching, and web-based modules extend learning beyond the classroom, provide real-time cues, and enable self-monitoring (e.g., photo-based food logs, stepwise goals). Systematic reviews in young adults show that well-designed eHealth programmes can improve diet behaviours, particularly when they incorporate behaviour-change techniques (action planning, prompts, feedback) and interactive features rather than static content (Hutchesson et al., 2015). For health professionals, e-learning platforms support scalable training in counselling, motivational interviewing, and dietary guidelines, performing at least as well as traditional formats while improving flexibility and reach (Vaona et al., 2018). Programmes should address digital equity (device access, data costs) and safeguard privacy.

Implementation, evaluation, and equity

Robust implementation closes the gap between efficacy and real-world impact. Key practices include: (i) fidelity monitoring of core components (e.g., number and quality of cooking sessions), (ii) mixed-methods evaluation that pairs diet quality indices (e.g., servings of fruit/vegetables, whole-grain intake, discretionary foods) with behavioural mediators (self-efficacy, food agency), and (iii) attention to reach and representativeness across age, gender, income, and cultural groups. Cost and feasibility should be tracked alongside outcomes to inform scale-up. Behaviour-change

frameworks encourage iterative adaptation based on local feedback while preserving the active ingredients identified a priori (Michie et al., 2011; Contento, 2016).

Putting it together: an integrated model

The most promising programmes blend components: a brief theory-grounded curriculum to build “why” and “how,” gamified and mobile supports to sustain motivation, hands-on cooking and sensory exploration to build capability, and community partnerships to address opportunity (affordability and access). When these layers are aligned, studies report meaningful improvements in knowledge, food choice, and self-efficacy, with early signals for better diet quality and weight-related outcomes over time (DeSmet et al., 2014; Reicks et al., 2014; Hutchesson et al., 2015; Vaona et al., 2018; Contento, 2016; Reverdy et al., 2008).

4.5 Evaluating Nutritional Awareness

Evaluating nutritional awareness is essential for determining whether nutrition education truly changes what people know, believe, and do. Robust assessment clarifies which messages are understood, where misconceptions persist, and how knowledge links to dietary choices and health. Effective evaluation blends psychometrically sound questionnaires with complementary behavioural and biological indicators, and increasingly leverages digital technologies to capture learning and real-world decisions.

Rationale and core constructs

Nutritional awareness encompasses factual knowledge (e.g., food sources of nutrients, dietary recommendations), procedural knowledge (e.g., interpreting labels, planning balanced meals), and conditional knowledge (when and why to apply skills). Because knowledge is only one driver of behaviour, high-quality evaluations also consider attitudes, self-efficacy, and environmental constraints, and test whether higher awareness correlates with healthier intake patterns and biomarkers (Spronk et al., 2014).

Standardized questionnaires

The General Nutrition Knowledge Questionnaire (GNKQ) is one of the most widely used instruments, covering dietary recommendations, food choices, nutrient sources, and diet–disease links; it has demonstrated reliability and construct validity and has been adapted cross-culturally (Parmenter and Wardle, 1999). The revised GNKQ-R updated item content to reflect contemporary dietary guidance, improved readability, and refined subscales for adults in diverse settings (Kliemann et al., 2016). Other validated tools target specific populations or domains (e.g., athletes, caregivers), and systematic development typically includes item generation from guidelines, expert review, cognitive interviewing, pilot testing, and psychometric evaluation (Hendrie et al., 2008; Trakman et al., 2017).

Psychometric quality and modern measurement

Beyond internal consistency and test–retest reliability, contemporary studies apply item response theory and Rasch analysis to optimise item difficulty and discrimination, detect differential item functioning across languages or demographics, and enable computer-adaptive testing that shortens surveys without sacrificing precision (Trakman et al., 2017). Measurement invariance testing ensures that observed score differences reflect true knowledge gaps rather than translation or cultural artefacts (Hendrie et al., 2008).

Comprehensive KAP surveys

Knowledge, Attitudes, and Practices (KAP) frameworks extend beyond factual knowledge to capture beliefs, perceived barriers, and self-reported behaviours. Standardised guidance from the Food and Agriculture Organization provides templates for sampling, questionnaire design, piloting, and analysis to enhance comparability across programmes and countries (FAO, 2014). KAP surveys are particularly useful for community-based interventions where cultural norms and food environments strongly shape choices.

Linking knowledge to behaviour and health

To validate that “knowing more” translates into “doing better,” evaluations pair knowledge scores with objective outcomes: 24-hour recalls, food frequency questionnaires, or digital food logs for intake; anthropometry (BMI, waist circumference); and biomarkers such as lipids, glucose, or micronutrient status. Meta-analytic evidence shows modest but consistent associations between higher nutrition knowledge and healthier dietary patterns—greater fruit and vegetable intake, lower saturated fat, and improved overall diet quality (Spronk et al., 2014). These linkages strengthen causal inference when measured longitudinally and adjusted for sociodemographic factors.

Technology-enhanced assessment

Mobile and web platforms can deliver brief quizzes, label-reading tasks, and just-in-time challenges, capturing response accuracy and latency as additional indicators of mastery. Apps also facilitate ecological momentary assessment of food choices and can integrate barcode scans or photo-based logs; systematic reviews report that well-designed digital tools can improve diet knowledge and related behaviours, especially when feedback and goal-setting are embedded (Chen et al., 2017). Digital analytics (e.g., item-level performance, completion rates) support rapid iteration of educational content.

Equity, culture, and context

Nutritional awareness is shaped by age, education, income, food literacy, and cultural foodways. Evaluations should incorporate plain-language items, visual aids, and context-specific examples (e.g., staple foods, customary portions), and should document reach and performance across subgroups to detect equity gaps (Hendrie et al., 2008; FAO, 2014). Translation/back-translation and cognitive interviewing reduce misinterpretation, while differential item functioning tests help ensure fair scoring across languages and cultures (Trakman et al., 2017).

Quality assurance and reporting

Strong studies preregister protocols; report reliability, validity, and measurement invariance; justify scoring rules and cut-points; and disclose limitations such as social desirability, guessing, and selection bias. When feasible, mixed-methods designs add explanatory depth by exploring why certain misconceptions persist despite instruction (Hendrie et al., 2008).

Putting it together

A multi-method strategy—validated questionnaires (GNKQ/GNKQ-R or population-specific tools), KAP modules, objective diet and biomarker endpoints, and technology-enabled task performance—offers a comprehensive view of nutritional awareness and its consequences. Such designs enable programmes to refine messages, tailor supports for subgroups, and demonstrate meaningful links from learning to healthier eating and improved clinical markers (Parmenter and Wardle, 1999; Kliemann et al., 2016; Spronk et al., 2014; FAO, 2014; Hendrie et al., 2008; Trakman et al., 2017; Chen et al., 2017.

4.6 Effectiveness of Innovative Nutrition Interventions

The effectiveness of innovative nutrition interventions has been examined across diverse populations and delivery settings, showing that well-designed programs can improve knowledge, shift food choices, and yield measurable clinical gains—although effects vary by context, intensity, and follow-up duration. Below, evidence is synthesized across major approaches, with attention to mechanisms, implementation considerations, and gaps that limit generalizability.

Technology-enhanced interventions

Digital health tools (mobile apps, portals, telehealth) and game-based formats can raise engagement and shorten feedback loops between behavior and reinforcement. A meta-analysis of board games for health reported a large pooled effect on health-related knowledge and small-to-moderate effects on behaviors and biological indicators, suggesting that structured play can translate learning into action when

games embed clear goals and feedback (Gauthier, 2019). Broader scoping and meta-analytic work on technology-assisted interventions indicates that interactive platforms are consistently effective for weight-related outcomes but show heterogeneous effects on diet quality, often due to short intervention windows, nonstandardized dietary endpoints, and limited personalization (Chew et al., 2023). Program features that strengthen impact include adaptive goal-setting, just-in-time prompts, and integration with human coaching; conversely, high user burden and notification fatigue undermine adherence and dilute effects over time (Chew et al., 2023).

Targeted and life-course approaches

Interventions aligned to critical windows (preconception, pregnancy, infancy, adolescence) demonstrate outsized returns relative to later-life programs. Balanced energy-protein supplementation during pregnancy reduces low birthweight and improves birth outcomes, underscoring the value of combining nutrition education with a concrete, context-appropriate supplement strategy (Lassi et al., 2021). Digital ecosystems that curate evidence-based resources for parents and practitioners can scale such approaches; early evaluations of integrated platforms report strong feasibility and user engagement, setting the stage for trials powered on growth and feeding outcomes (Øverby et al., 2023). In adolescence, multi-component “food literacy” models that blend workshops, experiential tasks, and digital elements improve nutrition knowledge, reduce emotional eating, and strengthen self-regulation—mechanisms that plausibly sustain healthier choices under stress and peer influence (Mancone et al., 2024).

Chronic disease management

Among adults with diet-related chronic conditions, digitally delivered dietary counseling and monitoring produce modest but clinically meaningful improvements in Mediterranean diet adherence, fruit and vegetable intake, sodium reduction, anthropometrics, and glycemic control—effects consistent with iterative self-monitoring and remote accountability (Barnett et al., 2023). These findings support embedding nutrition modules within disease management pathways, while emphasizing the need

for longer follow-up and cost-effectiveness analyses to inform reimbursement (Barnett et al., 2023).

Community and social behavior change strategies

Nutrition social and behavior change communication (NSBCC) programs that operate through community structures—using repeated, multi-channel messaging with practical demonstrations—have demonstrated increases in exclusive breastfeeding and improvements in child growth indicators. Effect sizes are strongest where communication is paired with enablers (e.g., food access, time-saving supports), highlighting that knowledge alone is insufficient in resource-constrained settings (Mahumud et al., 2022).

Mechanisms of action and effect modifiers

Across modalities, several drivers recur: (1) salience and repetition (nudges, reminders), (2) self-efficacy and skills (label reading, budgeting, meal prep), (3) social reinforcement (peers, families, community leaders), and (4) structural support (healthy defaults, access). Intervention "dose," tailoring to cultural and literacy contexts, and baseline nutrition literacy consistently moderate outcomes (Chew et al., 2023; Mancone et al., 2024). Programs that pair education with tangible resources (subsidies, supplements, or food boxes) show larger and more durable effects (Lassi et al., 2021).

Implementation, equity, and sustainability

Effectiveness depends on fidelity (delivery as intended), reach (who participates), and maintenance (post-trial continuation). Digital divides, language barriers, and variability in food environments can widen inequities if not proactively addressed through offline options, multilingual content, and linkage to social supports (Chew et al., 2023; Mahumud et al., 2022). For scalability, hybrid designs that combine automated features with light-touch human support appear most cost-efficient while preserving adherence (Barnett et al., 2023). Routine monitoring should incorporate equity-stratified metrics and user-centered process data (engagement time, prompt responsiveness) to guide mid-course corrections (Øverby et al., 2023).

Measurement and outcomes

Short-term knowledge gains are common, but sustained dietary change requires longer follow-up and harmonized endpoints (e.g., standardized diet quality indices, objective purchase or intake data). Where feasible, pairing self-report with biomarkers or connected-device measures improves validity. Maintenance effects are strongest when post-intervention “booster” touchpoints and environmental supports are in place (Gauthier, 2019; Barnett et al., 2023).

Overall synthesis

Innovative nutrition interventions—particularly those that are interactive, tailored, and paired with structural enablers—can improve knowledge and produce meaningful behavioral and clinical changes. The strongest evidence supports life-course targeting, blended human-digital delivery, and community-embedded SBCC models. Future work should prioritize longer trials with equity-sensitive designs, cost-effectiveness analyses, and interoperability with health and social systems to sustain impact at scale (Chew et al., 2023; Barnett et al., 2023; Mahumud et al., 2022; Lassi et al., 2021; Øverby et al., 2023; Mancone et al., 2024; Gauthier, 2019).

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