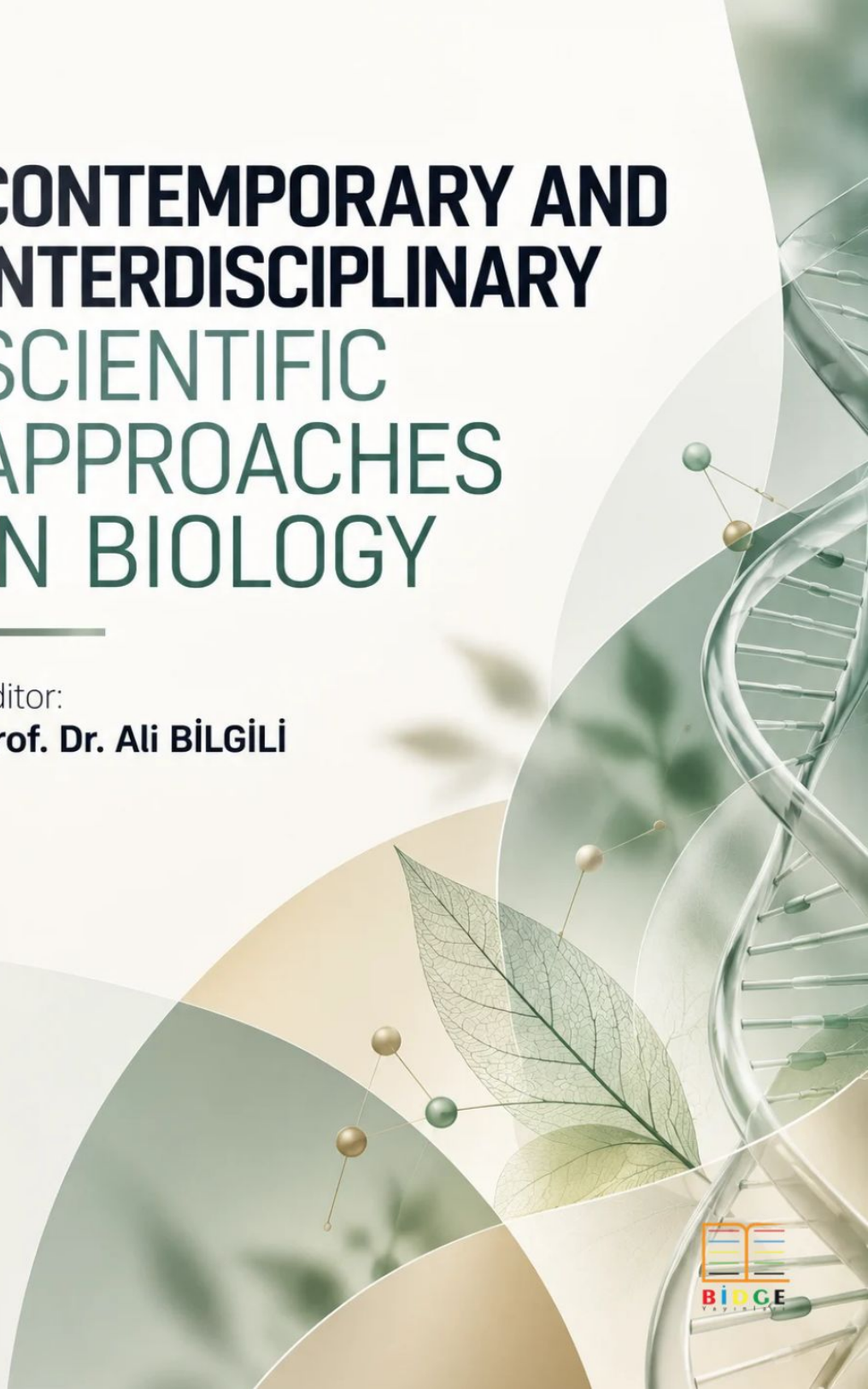
CONTEMPORARY AND INTERDISCIPLINARY SCIENTIFIC APPROACHES IN BIOLOGY

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CHAPTER 1

ALGAE-DERIVED BIOACTIVE COMPOUNDS IN COMBATING ANTIBIOTIC RESISTANCE: CHEMICAL DIVERSITY, ANTIMICROBIAL MECHANISMS, AND THERAPEUTIC POTENTIAL

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Introduction

Antimicrobial resistance (AMR), including resistance to antibiotics, is a major global health challenge because it can make bacterial infections more difficult to treat and increase healthcare

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use, treatment costs, and mortality. The global burden of bacterial AMR was substantial in 2019, and inappropriate or unnecessary antibiotic use contributes to selection pressure that favours resistant organisms (Antimicrobial Resistance Collaborators, 2022, p. 629; Ventola, 2015, pp. 277–278). The World Health Organization's Bacterial Priority Pathogens List identifies resistant bacterial groups for which research and development of new interventions are particularly important (World Health Organization [WHO], 2024, p. x).

The problem is not limited to the emergence of resistance mechanisms in individual bacterial cells. Biofilm growth, altered metabolic states, persister cells, and restricted antibiotic penetration can contribute to reduced treatment susceptibility or tolerance. These phenomena are related but not interchangeable: antibiotic resistance generally denotes the ability to grow at antibiotic concentrations that inhibit susceptible organisms, whereas tolerance refers to survival during antibiotic exposure without necessarily increasing the minimum inhibitory concentration (MIC). Biofilm-associated tolerance may coexist with genetically encoded resistance, but biofilm formation alone does not establish that a strain is antibiotic-resistant (Hall & Mah, 2017, pp. 277–278; Liu et al., 2024, pp. 1, 4).

Natural products have contributed substantially to drug discovery, including the discovery of antimicrobial agents and compounds that serve as starting points for medicinal chemistry. Modern natural-product research combines biological screening with chemical characterization, isolation, structural elucidation, and optimization of promising molecules (Atanasov et al., 2021, pp. 200–202; Newman & Cragg, 2020, pp. 770–771). Algae are chemically diverse organisms and have attracted attention as sources of candidate antimicrobial compounds. Evidence includes direct antibacterial screening, antibiofilm investigations, and studies examining whether algal extracts can improve the activity of

selected antibiotics (Behzadnia et al., 2024, p. 1; EL-Sayed et al., 2023, pp. 3, 7; Silva et al., 2020, pp. 1–3; Zuorro et al., 2024, p. 1).

This chapter uses the term algae-derived compounds as an inclusive term because the literature discussed here includes marine macroalgae, freshwater filamentous algae, and a diatom. These groups differ taxonomically and ecologically: macroalgae or seaweeds are macroscopic marine algae, whereas *Spirogyra* and *Vaucheria* are freshwater-associated filamentous algae in many settings, and *Gomphonema olivaceum* is a microscopic diatom. They should not all be described as marine macroalgae. The chapter evaluates their chemical diversity and reported biological activities while distinguishing evidence of antimicrobial activity from evidence that a compound modifies antibiotic resistance.

The available findings should be interpreted as preclinical research evidence rather than proof of clinical efficacy. An extract that inhibits bacterial growth in vitro does not, by itself, demonstrate reversal of antibiotic resistance, safety in humans, or suitability for therapeutic use. Stronger conclusions require reproducible methods, well-characterized resistant isolates, mechanistic experiments, toxicity assessment, and ultimately appropriate in vivo and clinical evaluation.

Algae-Derived Bioactive Compounds

The chemical profile of an algal sample depends on its taxon, habitat, season, physiological state, cultivation conditions, harvesting and storage procedures, and extraction method. Major classes investigated for antimicrobial potential include phenolic compounds, fatty acids and other lipids, polysaccharides, pigments, terpenoids, and sterols. Chemical composition and biological activity should therefore be assessed together rather than inferred from taxonomic identity or total compound measurements alone

(Cotas et al., 2020, pp. 1, 2, 8, 9; Shannon & Abu-Ghannam, 2016, p. 2; Silva et al., 2020, pp. 9–12; Zuorro et al., 2024, pp. 12–13).

Phenolic compounds

Phenolic compounds are frequently investigated in algal extracts because their chemical structures can interact with proteins, membranes, and other cellular targets. Brown seaweeds are notable for phlorotannins, while phenolic acids and other phenolic-like compounds have also been reported in different algal materials. Some phenolics exhibit antimicrobial effects in experimental systems, but activity varies with molecular structure, concentration, bacterial species, and assay conditions (Cotas et al., 2020, pp. 2–3; Cushnie & Lamb, 2011, pp. 101–102; Daglia, 2012, pp. 176–178).

Total phenolic content is a useful screening parameter, not proof that phenolics are responsible for an observed antibacterial effect. Correlations between total phenolic content and activity can be confounded by other extract constituents and by differences in extraction yield. Where possible, profiling by chromatographic and spectrometric methods, testing isolated compounds, and conducting structure–activity analyses are needed to identify active constituents. Studies of *Vaucheria* species that combine phenolic measurements with antioxidant and antimicrobial testing are relevant examples of this integrated approach, but they do not by themselves demonstrate antibiotic-resistance reversal (Uç & Pabuçcu, 2024, pp. 16–17; Uç & Pabuçcu, 2026, pp. 1396–1397).

Fatty acids and other lipids

Certain free fatty acids can affect bacterial membranes, and their antimicrobial properties depend on chain length, degree of unsaturation, concentration, and the microorganism tested (Desbois & Smith, 2010, pp. 1632–1634). However, fatty-acid profiles are not equivalent to antimicrobial activity: detecting a fatty acid in an algal

sample does not establish that it is present at an active concentration or that it accounts for an extract's effect.

Chemical studies of the freshwater green alga *Cladophora fracta var. intricata* and the diatom *Gomphonema olivaceum* illustrate the value of characterizing algal lipids and related constituents (Demiriz Yücer & Pabuçcu, 2023, pp. 75–76; Demiriz Yücer & Pabuçcu, 2024, pp. 90–92). These studies support chemical-diversity research but should not be cited as direct demonstrations of activity against antibiotic-resistant bacteria unless such experiments were explicitly performed.

Polysaccharides

Algal polysaccharides are of interest in pharmaceutical, food, and biomaterials research. Their structures vary substantially across algal groups. Fucoidans are characteristic sulfated polysaccharides associated particularly with brown algae; agar and carrageenans are obtained from red algae; and alginates are characteristic polysaccharides of brown algae. The biological properties of these materials depend on features such as molecular mass, monosaccharide composition, sulfation pattern, purity, and formulation (Silva et al., 2020, pp. 10–11).

Some algal polysaccharides or polysaccharide-rich fractions have been investigated for antibiofilm or other biological effects, but effects cannot be generalized to all polysaccharides (Behzadnia et al., 2024, p. 10). A reduction in biofilm biomass may reflect impaired adhesion, altered matrix production, reduced bacterial growth, or a combination of processes. Appropriate controls and independent measures of bacterial viability are needed to distinguish these possibilities.

Pigments and other metabolites

Chlorophylls, carotenoids, terpenoids, sterols, and other low-molecular-weight metabolites contribute to algal chemical diversity and may be relevant to antimicrobial discovery. Nevertheless, antioxidant and antimicrobial activities are distinct endpoints. An extract's radical-scavenging activity does not establish that it kills bacteria or induces oxidative stress in them. Claims about a particular pigment or metabolite should therefore be supported by studies that directly test the proposed activity and, where possible, the purified compound (Gupta & Abu-Ghannam, 2011, pp. 317–319; Shannon & Abu-Ghannam, 2016, pp. 2, 5, 6; Zuorro et al., 2024, pp. 1, 9, 11).

Extraction and chemical characterization

Extraction procedures strongly influence the compounds recovered from algal biomass. Water, ethanol, methanol, acetone, and non-polar solvents differ in polarity and selectivity; consequently, extracts from the same species can have different chemical profiles and biological activities. Solvent residues, extraction yield, biomass processing, and the use of appropriate solvent controls must also be considered. Studies of *Spirogyra aequinoctialis* using different solvents illustrate why extraction conditions should be reported and compared carefully (Pabuçcu & Demiriz Yücer, 2022, pp. 928–930; Silva et al., 2020, pp. 3, 21, 22).

For reliable interpretation, studies should report taxonomic identification and voucher or culture information where available, collection or cultivation conditions, biomass processing, extraction yield, solvent controls, chemical characterization, test strains, inoculum preparation, and assay conditions. Activity-guided fractionation and metabolomic approaches can help connect biological effects with particular chemical constituents.

Mechanisms Relevant to Antibiotic Resistance and Treatment Tolerance

Algae-derived compounds are being explored not only for direct antimicrobial effects but also for potential effects on processes associated with bacterial survival, pathogenicity, and reduced treatment susceptibility. The principal areas of interest include membrane effects, biofilm formation, quorum sensing, efflux pumps, and interactions with existing antibiotics. The strength of evidence varies by compound and study; these mechanisms should be presented as demonstrated only when directly tested.

Effects on bacterial membranes and cell walls

The bacterial cell membrane maintains ion gradients and cellular homeostasis, while the cell wall provides structural support. Some antimicrobial compounds can disrupt membrane permeability or affect cell-wall-associated processes, ultimately impairing viability. Free fatty acids are one class of molecules for which membrane-associated antibacterial mechanisms have been investigated (Desbois & Smith, 2010, pp. 1633–1634).

However, a growth-inhibition zone or a low MIC does not identify the mechanism responsible. In crude algal extracts, membrane-permeability assays, measurements of intracellular leakage, microscopy, and testing of purified fractions may be required to establish whether membrane damage contributes to activity. It is also important not to assume that a membrane-active compound will automatically overcome resistance to antibiotics with unrelated targets.

Biofilm formation and biofilm-associated tolerance

Biofilms are microbial communities embedded in an extracellular matrix and attached to a surface or to one another. Their physiology can differ from that of planktonic cells. Matrix

properties, slow growth, altered gene expression, persister cells, and limited penetration or activity of some antibiotics can contribute to reduced treatment susceptibility (Flemming et al., 2016, pp. 563, 571, 572; Hall & Mah, 2017, pp. 277–278; Liu et al., 2024, pp. 1, 4).

Marine algae-derived compounds, including certain phenolics, lipids, pigments, and sulfated polysaccharides, have been discussed as candidate antibiofilm agents (Behzadnia et al., 2024, pp. 10–11). Nevertheless, preventing initial biofilm formation is different from disrupting an established biofilm. Biofilm biomass assays should ideally be paired with viable-cell measurements and, where relevant, microscopy or matrix analysis. A reduction in biomass alone does not establish bacterial killing or reversal of antibiotic resistance.

Quorum sensing and virulence-associated traits

Quorum sensing is a cell-density-dependent communication system through which bacteria regulate gene expression in response to signalling molecules. Depending on the organism, quorum-sensing pathways can influence biofilm development, motility, extracellular enzyme production, and other virulence-associated traits. Interfering with these pathways may reduce selected pathogenic traits without necessarily inhibiting bacterial growth (Behzadnia et al., 2024, pp. 3–4).

An antibiofilm effect does not by itself prove quorum-sensing inhibition. Reduced bacterial growth, altered adhesion, and changes in matrix production can produce similar observations. Evidence for quorum-sensing interference is stronger when reporter strains, signalling assays, gene-expression measurements, and purified compounds are used. Reviews of algal antibiofilm compounds and broader quorum-quenching strategies identify quorum sensing as a promising research area, but compound-specific

claims should remain proportional to the experimental evidence (Behzadnia et al., 2024, pp. 3, 4, 10; D'Aquila et al., 2024, pp. 1–3).

Efflux pumps

Efflux pumps are membrane-associated transport systems that can export antibiotics and other compounds from bacterial cells. Depending on the organism and pump, their activity can contribute to intrinsic or acquired resistance and may interact with other cellular processes (Blair et al., 2015, pp. 43–45; Ren et al., 2024, pp. 1–2).

Algae-derived compounds could be screened as potential efflux-pump modulators, but this possibility should not be described as an established property of algal extracts without direct evidence. Useful approaches include measuring intracellular accumulation of a suitable probe or antibiotic, assessing efflux activity, and using appropriate pump-deficient or pump-overexpressing strains where available. Cytotoxicity and non-specific membrane disruption must be considered because either can confound apparent efflux inhibition.

Interactions with existing antibiotics

Combination studies are among the most directly relevant approaches for assessing whether an algal extract may modify antibiotic activity. EL-Sayed et al. (2023, pp. 3, 7) investigated methanolic *Ulva lactuca* extract alone and in combination with selected antibiotics against *Klebsiella pneumoniae* RCMB 003 (1) ATCC 13883, described by the authors as multidrug-resistant. The authors reported enhanced inhibition for particular combinations, including a combination with gentamicin. This is a useful proof-of-concept study, but the finding should not be generalized to all *Ulva* extracts, antibiotics, or bacterial isolates.

Broth microdilution assays and fractional inhibitory concentration indices (FICI), together with time-kill experiments,

can help characterize combination effects (Balouiri et al., 2016, pp. 73, 77; Cushnie & Lamb, 2011, pp. 103–104). The assay method and interpretation thresholds should be stated explicitly. Agar diffusion alone can be difficult to interpret for synergy because diffusion rates differ between crude extracts and antibiotics. Further work should use chemically characterized fractions, multiple well-characterized resistant isolates, appropriate controls, and reproducible quantitative methods.

Even when a combination enhances inhibition in vitro, it does not automatically demonstrate that antibiotic doses can safely be reduced in patients. Such conclusions require pharmacological, toxicological, and clinical evidence.

Evidence from Algal Research

Published studies provide several types of evidence, which should be distinguished according to what they actually demonstrate. Antibacterial screening supports direct activity under the tested conditions; chemical-composition studies identify candidate constituents; antibiofilm studies examine a specific microbial phenotype; and combination studies may provide evidence of antibiotic-potentiating effects. These categories are complementary but not interchangeable.

The study of *Spirogyra aequinoctialis* by Pabuçcu and Demiriz Yücer (2022, pp. 928–930) examined antibacterial and antifungal activities of extracts prepared with different solvents. It supports the value of solvent-dependent screening of freshwater algal material, but it should not be presented as direct evidence of activity against antibiotic resistance unless resistant isolates and resistance-related endpoints were tested.

Studies of *Gomphonema olivaceum* and *Cladophora fracta var. intricata* characterized fatty acids and, in the latter case, vitamins and antioxidant properties (Demiriz Yücer & Pabuçcu, 2023, pp. 75–

76; Demiriz Yücer & Pabuçcu, 2024, pp. 91–92). These findings are relevant to algal chemical diversity and candidate discovery, but chemical detection and antioxidant assays do not establish antibacterial activity or resistance modification.

Uç and Pabuçcu (2024, pp. 16–18) investigated antioxidant and antibacterial properties of *Vaucheria frigida*, and Uç and Pabuçcu (2026, pp. 1396–1397) reported antioxidant and antimicrobial activities alongside phenolic composition for *Vaucheria aversa*. These studies are relevant examples of combining chemical and biological characterization in freshwater algae. Their results should be interpreted within the organisms, extracts, and test conditions investigated; they do not alone establish a mechanism for reversing antibiotic resistance.

The *Ulva lactuca* study by EL-Sayed et al. (2023, pp. 3, 7) is more directly related to antibiotic-combination research because it tested the extract in combination with selected antibiotics against *K. pneumoniae* RCMB 003 (1) ATCC 13883, described by the authors as multidrug-resistant. Its findings justify further investigation of algal extracts as potential antibiotic-potentiating candidates, while requiring replication, improved chemical attribution, and evaluation across additional isolates and methods.

The review of marine algal antibiofilm compounds synthesizes candidate compound classes and possible mechanisms, including adhesion, extracellular matrix production, and quorum sensing (Behzadnia et al., 2024, pp. 9–11). Broader reviews cover macroalgal antimicrobial compounds and extraction methods (Silva et al., 2020, pp. 9–12) and algal antimicrobial discovery (Zuorro et al., 2024, p. 1). Because reviews summarize heterogeneous studies, individual mechanistic claims should be traced to the primary experiments where possible. Research on antiproliferative effects of *Pseudopediastrum boryanum* extracts, for example, may inform the broader need for cellular safety and selectivity assessment, but it is

not direct evidence of anti-AMR activity (Demiriz Yücer, 2024, pp. 1027–1029).

Methodological Limitations and Future Directions

Several limitations currently constrain translation of algae-derived bioactive compounds into antimicrobial applications. First, differences in taxonomic identification, habitat, season, cultivation, biomass processing, extraction solvents, and test methods can make results difficult to compare. Standardized reporting and quality control are essential. Second, crude extracts contain mixtures of compounds, so activity cannot reliably be attributed to a particular molecule without fractionation, purification, and chemical analysis (Atanasov et al., 2021, pp. 201–202; Silva et al., 2020, pp. 3, 9, 27).

Third, studies should use clinically relevant and well-characterized bacterial strains. Where the research question concerns antibiotic resistance, investigators should specify the resistance phenotype or genotype and use appropriate susceptibility testing. Priority groups include methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, third-generation-cephalosporin-resistant Enterobacterales, carbapenem-resistant *Acinetobacter baumannii*, and carbapenem-resistant *Pseudomonas aeruginosa*, consistent with the aims of the WHO priority-pathogen framework (WHO, 2024, pp. x–xi).

Fourth, mechanistic studies should match the claim being made. MIC and minimum bactericidal concentration measurements assess different endpoints (Balouiri et al., 2016, pp. 75–77); biofilm biomass, viable-cell counts, quorum-sensing reporters, efflux assays, and time-kill experiments answer different questions. Appropriate positive, negative, solvent, and growth controls are necessary. For combination studies, results should be reported for each tested pairing rather than generalized from a single successful combination.

Finally, safety and pharmacological feasibility require early attention. Cytotoxicity, haemolysis where relevant, selectivity indices, stability, solubility, bioavailability, metabolism, and pharmacokinetic properties may determine whether a promising in vitro hit can be developed further. In vivo studies and clinical investigations are needed before therapeutic claims can be made. Natural origin should not be equated with safety.

A practical research pathway is therefore to (1) authenticate and document the algal material; (2) prepare standardized extracts; (3) conduct reproducible antimicrobial screening; (4) characterize the chemistry of active extracts; (5) fractionate and identify active constituents; (6) validate proposed mechanisms; (7) test combinations against characterized resistant isolates; and (8) assess toxicity and preclinical feasibility. This workflow can help distinguish reproducible lead compounds from nonspecific or assay-dependent effects.

Conclusion

Algae-derived bioactive compounds represent a promising source of candidate molecules for antimicrobial research and the development of complementary strategies against antibiotic-resistant bacteria. Their potential applications extend beyond direct antimicrobial activity to include antibiofilm effects, interference with bacterial communication systems, and possible enhancement of antibiotic efficacy. However, these effects vary according to algal taxon, chemical composition, extraction procedures, target microorganisms, and experimental conditions. Antimicrobial activity alone should not be interpreted as evidence of resistance reversal or clinical efficacy. Future studies should prioritize chemical characterization, compound-level validation, standardized antimicrobial susceptibility testing, investigations using well-characterized resistant clinical isolates, antibiotic-combination

studies, and systematic toxicity and selectivity assessments. Through these approaches, algae-derived compounds may provide valuable starting points for the discovery of new antimicrobial agents and resistance-modifying adjuncts, although their clinical relevance must be established through rigorous preclinical and clinical evaluation.

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CHAPTER 2

PLANT-DERIVED CARBON DOTS FOR DRUG DELIVERY AND BIOPHARMACEUTICAL APPLICATIONS

ASLIHAN CESUR TURGUT[1]

Introduction

An active compound can produce a promising response in a biological assay without being delivered effectively by a pharmaceutical formulation. The relevant delivery question is whether the final preparation provides an appropriate exposure at the intended site for a useful duration. For botanical carbon dots, that question involves the associated drug, the carbon-containing carrier, and any soluble constituents that remain after preparation. Each can contribute to the observed response and each may follow a different biological path.

There are also two distinct pharmaceutical roles to consider. A dot may have intrinsic activity after a botanical precursor has been transformed, or it may carry a separately supplied therapeutic compound. The distinction is practical: the dose and molecular identity of a loaded drug can be measured independently, whereas

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activity assigned to a carbonized botanical preparation requires evidence about the active fraction. A study of Plantago major-derived dots that examined purification alongside biological responses illustrates the importance of defining the preparation being tested, without establishing a general drug-carrier function for such materials (Altinkaya, 2025, p. 1).

This chapter examines drug loading, release, barrier transport, tissue distribution, and elimination as connected formulation questions. It is a critical narrative review of selected primary research rather than a systematic assessment of all reported carbon-dot carriers. Whole-plant-derived examples and isolated-phytochemical-derived systems are identified separately. Studies of synthetic or carbohydrate-derived dots are included where they provide mechanistic or analytical comparisons; their results are not treated as direct validation of medicinal-plant-derived formulations.

The available examples mainly address experimental formulations and preclinical models. The proposed evaluation steps are intended to help design interpretable studies, with the required evidence determined by the route and target. A local skin formulation may benefit from retention, whereas a systemic preparation must establish absorption or tissue exposure beyond the initial barrier. The same material characteristic can therefore have different implications for different delivery objectives.

Defining a Botanical Carbon-Dot Formulation

Precursor class and carrier identity

The carbon-dot designation includes structurally diverse products. Precursor-dependent classifications distinguish materials with different carbonized and polymer-like features, so fluorescence alone is insufficient to establish a specific subtype (Zeng et al., 2021, p. 1). This chapter uses carbon dot as the broad designation and retains more specific terminology only when describing a

publication that used it. Quantum confinement is not assumed for every botanical dispersion.

The botanical contribution should also be stated precisely. A carrier prepared from plant tissue or extract differs from one synthesized using an isolated phytochemical. A drug added after synthesis is a cargo rather than proof that the same molecule survived carbonization. For every delivery preparation, the proposed record should identify the plant species or molecular precursor, any co-precursor, the purified carrier, the added drug, and the procedure used to obtain the final formulation. A change in any of these elements may change the exposure being evaluated.

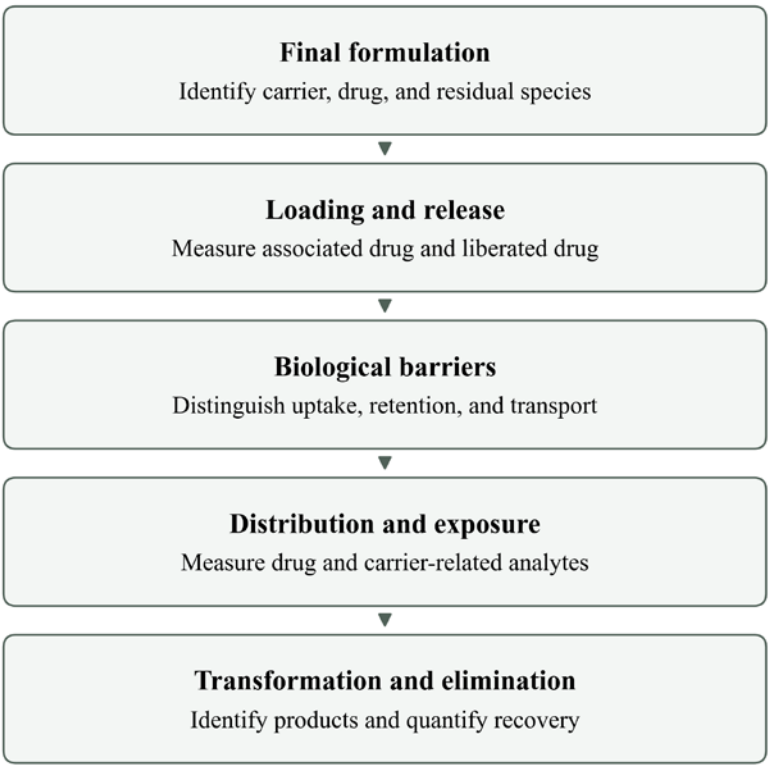
Purity and characterization of the administered material

Purification is part of formulation definition. A fluorescent reaction mixture may include molecular species as well as larger carbon-containing products, and these components can interact during separation. Research on purification and molecular fluorophores demonstrates why an optical signal must be interpreted with compositional evidence (Otten et al., 2022, p. 6148). This issue becomes particularly important when fluorescence is used to track an administered preparation.

The proposed characterization should therefore proceed beyond the unloaded stock. Dry-state morphology, hydrodynamic size, surface properties, drug integrity, and aggregation should be assessed for the final drug-loaded material under relevant medium conditions. Free drug and removable reaction products should be quantified or otherwise bounded by a validated separation method. Carrier concentration, drug concentration, and analytical recovery should remain identifiable throughout the study. These measurements provide the basis for interpreting the loading, transport, and fate experiments considered in the following sections.

Figure 1 summarizes the questions that connect formulation identity to biological fate. The arrows indicate a proposed assessment sequence rather than an experimentally established common pathway for all carbon dots. Drug and carrier-related measurements remain distinct at each stage.

Figure 1. Proposed assessment sequence for a drug-loaded botanical carbon-dot formulation.



Source: Conceptual assessment scheme developed by the author, informed by experimental studies of release, barrier transport, distribution, and clearance (Liao et al., 2021, p. 1; Licciardello et al., 2018, p. 9880; Modi & Anderson, 2013, p. 3076; Seven et al., 2021, p. 3942). Arrows represent assessment steps rather than a universal biological pathway.

Drug Loading and Controlled Release

Loading strategy and quantitative definitions

A biopharmaceutical assessment asks whether a formulation delivers an active substance to its intended site at an appropriate concentration and time. For botanical carbon dots, this requires separating the activity of the carrier from that of an externally loaded drug. Surface adsorption, covalent attachment, and incorporation into a larger composite should be described according to the preparation and analytical evidence. A carbonized phytochemical is not automatically equivalent to a carrier loaded with the intact parent compound. The proposed development strategy in this chapter therefore begins with identifying the drug, the purified carrier, and the final drug–carrier preparation as separate analytical samples.

Two quantities are particularly useful: loading efficiency, calculated as the mass of associated drug divided by the initially supplied drug mass, and loading content, calculated as the associated drug mass divided by the mass of the final drug–carrier material. Both can be expressed as percentages, but a drug-to-carrier mass ratio has a different denominator. These definitions must accompany reported values. In a curcumin–carbon-nanodot study, adsorption efficiencies of 91% and 82% coexisted with capacities of approximately 3.4–3.8% relative to carrier mass. This illustrates why efficient capture of the supplied drug does not necessarily mean that drug constitutes a large fraction of the administered formulation (Arvapalli et al., 2020, p. 8776).

For future botanical formulations, the author proposes validating drug quantification after removal of free drug, with recovery experiments and controls for carbon-dot interference. Chromatography can provide an independent measurement when fluorescence or ultraviolet absorbance overlaps with the carrier signal. A formulation record should include carrier mass, drug mass,

purification losses, and the stability of the associated drug during storage and incubation. Biological comparisons should use matched drug doses and report the accompanying carrier dose; otherwise, apparent improvements may reflect unequal exposure.

Botanical examples and acid-dependent release

Direct botanical examples support further investigation. Carbon dots prepared from bamboo leaves were used to carry curcumin, with enhanced release under a pH 5.5 test condition and greater cytotoxic responses in cultured cancer cells than those obtained with free curcumin. The reported experimental scope supports an in vitro delivery application, while a claim of improved systemic bioavailability would require additional pharmacokinetic measurements (Saini et al., 2025, p. 2282).

In another study, carbon dots derived from *Artemisia argyi* were loaded with doxorubicin. Reported loading efficiency and loading content were 66.4% and 57.1%, respectively. At 56 hours, cumulative release was 56.55% at pH 4.0 and 6.81% at pH 7.4, and the formulation was evaluated in a mouse hepatocellular carcinoma model. These findings establish acid-dependent release for the tested preparation; pH 4.0 should be interpreted as a strongly acidic experimental condition rather than assumed to represent the extracellular environment of every tumor. The animal results remain preclinical evidence (Jia et al., 2025, p. 37645).

Taken together, these examples motivate a formulation question: does drug association improve exposure under the intended route and biological conditions? The answer should be based on the final loaded preparation. Particle size, surface charge, aggregation, and drug integrity should be reassessed after loading rather than carried over from measurements of the unloaded dots.

Release testing and interpretation of kinetics

A slow concentration increase outside a dialysis membrane does not by itself demonstrate slow release from the carrier. Dynamic dialysis contains two sequential processes: liberation of drug from the formulation and transport of unbound drug through the membrane. Experimental and modeling work on nanoparticle release has shown that membrane transport and drug binding can distort the apparent release profile. Although that work examined other nanoparticle systems, its methodological lesson is relevant to carbon-dot formulations (Modi & Anderson, 2013, p. 3076).

The proposed assessment should therefore include a free-drug membrane control, recovery from the apparatus, suitable receiving-medium capacity, and correction for sampling and replacement volumes. Drug stability should be measured over the full experiment. Release profiles at different pH values should use comparable assay conditions, and a physiologically relevant protein-containing medium should be considered when feasible. Testing more than one carrier concentration can help identify concentration-dependent binding or aggregation.

Kinetic fitting should be used to summarize observations and compare plausible explanations. A high correlation coefficient for a selected equation is insufficient to establish diffusion, erosion, or a specific bond-cleavage mechanism. For a controlled-release claim, the useful endpoint is reproducible, formulation-dependent release with an identified practical benefit. For a targeting claim, additional evidence must link release to exposure and response at the intended site.

Transport Across Biological Barriers

Cellular uptake, protein adsorption, and epithelial transport

Cellular fluorescence can show that a signal associates with or enters cells, but it does not establish passage through an epithelial or endothelial barrier. A barrier assessment should distinguish surface adhesion, intracellular retention, movement to the opposite compartment, and delivery of an active payload. For the botanical systems considered here, the author proposes combining microscopy with drug quantification in donor and receiver compartments and a measurement of barrier integrity. Cell viability, electrical resistance where applicable, and an independent permeability tracer can help identify transport caused by barrier injury.

Biological exposure also changes the interface presented by a carbon dot. In a study of four amine-passivated carbon-dot preparations, serum protein adsorption depended on surface properties and was associated with differences in macrophage uptake and viability. This is mechanistic evidence from nonbotanical formulations; it supports testing protein interactions in botanical candidates rather than assuming that botanical origin predicts their behavior in serum (Arezki et al., 2022, p. 14695). The final drug-loaded formulation should be examined because its surface can differ from that of the unloaded carrier.

Mucus and skin as route-specific barriers

Mucosal delivery illustrates a trade-off between interaction and movement. A surface that binds strongly to biological material may favor retention but also restrict access to underlying cells. Engineered carbon dots prepared from citric acid and polyethyleneimine were investigated with polyethylene glycol, zwitterionic, and thiol-containing modifications. Their performance in mucus models and an airway epithelial model differed, and improved movement through mucus did not uniformly produce

stronger gene expression. The study shows why barrier transit and successful cargo delivery require separate endpoints; it is not direct evidence for whole-plant-derived dots (Arca et al., 2025, p. 1).

For a botanical formulation intended for a mucosal route, the proposed evaluation should use relevant mucus composition and an epithelial system that reflects that route. Carrier-associated signal, released drug, epithelial transport, and the intended pharmacological response should be measured separately. Residence time may be desirable for a local treatment, whereas a systemic formulation requires evidence of absorption beyond the epithelial surface. These are different development objectives.

Skin delivery likewise needs an explicit target. Carbon dots synthesized from epigallocatechin-3-gallate were incorporated into a hydrogel and investigated in a mouse model of atopic dermatitis, including epidermal penetration and anti-inflammatory responses. This example connects phytochemical-derived dots with local biological activity, rather than demonstrating delivery of an independently loaded drug. Penetration in diseased mouse skin should not be generalized to intact human skin or taken as proof of systemic transdermal absorption (Han et al., 2024, p. 24308).

For topical development, the author proposes measuring retention in the relevant skin layers and passage into the receiving compartment of a validated diffusion experiment. Comparisons with free active compound, unloaded carrier, and the vehicle can distinguish formulation effects. The preferred outcome should match the product objective: local skin retention, delivery to a deeper tissue, or systemic absorption.

The blood–brain barrier and limits of mechanistic inference

Glucose-derived carbon dots conjugated to fluorescein were investigated for cargo delivery across the blood–brain barrier in zebrafish and rats. The same study used glucose-transporter-positive

and transporter-deficient yeast to examine uptake. The yeast findings support a transporter-related uptake hypothesis in that experimental system, but they do not independently establish the transport mechanism at the rat blood–brain barrier. Moreover, fluorescein delivery is a probe-based demonstration rather than evidence of therapeutic efficacy in neurological disease (Seven et al., 2021, p. 3942).

For botanical carbon dots, a brain-delivery claim would require evidence specific to the tested formulation. A proposed sequence is to establish barrier integrity in a relevant model, quantify transport of both carrier and payload, and then distinguish vascular association from brain-tissue exposure in vivo. An observed brain signal should be interpreted alongside label stability and appropriate controls. Botanical origin and a small dry-state particle diameter are insufficient substitutes for these measurements.

Biodistribution and Pharmacokinetic Evaluation

Measuring drug and carrier exposure separately

The biological fate of a loaded preparation can differ from that of its free drug or unloaded carrier. This distinction is especially important when drug loading changes surface properties. In a sucrose- and serine-derived carbon-dot formulation for doxorubicin delivery, drug loading changed the reported zeta potential from approximately −14 to +58 mV. The study evaluated antitumor effects in a mouse renal carcinoma model, while biodistribution imaging used a dye-loaded preparation. Consequently, the imaging construct and therapeutic formulation should not be assumed to have identical behavior solely because they share the same carbon-dot precursor (Azmi et al., 2025, p. 2751).

The proposed pharmacokinetic strategy should measure drug concentration over time and, where analytically feasible, distinguish carrier-associated drug from released drug. Area under the

concentration–time curve, elimination half-life, clearance, and distribution volume should be calculated from an appropriate sampling design and explicitly identified analyte. Comparisons should match administration route and drug dose. Carrier exposure should be reported independently, with a validated method and its recovery limits. A separation procedure that changes drug binding during sample processing can itself bias the apparent free-drug fraction.

Organ localization and the limits of imaging

Whole-body fluorescence is useful for locating a detectable signal, but signal intensity is not automatically proportional to the amount of intact carrier. Tissue attenuation, local chemical conditions, degradation, and release of a fluorescent label can complicate interpretation. Complementary labeling approaches can strengthen an assessment: a primary study combined optical labeling and copper-64 radiolabeling to investigate ultrasmall carbon dots and silicon nanoparticles in animals. Their surface characteristics affected biological behavior, reinforcing the need for formulation-specific measurements rather than a universal fate assigned to all small dots (Licciardello et al., 2018, p. 9880).

For future botanical studies, the author proposes sampling plasma, the target tissue, and plausible clearance organs at defined intervals, with tissue blanks and calibration appropriate to each matrix. A stable label should be checked under the same exposure conditions as the formulation. Tissue drug concentration and tissue carrier-associated signal answer different questions and should be reported as such. Improved target exposure should also be linked to an independently measured biological response. Localization alone cannot establish active targeting, intracellular release, or a favorable therapeutic margin.

Metabolism and Excretion

Renal and hepatobiliary pathways

Carbon-dot clearance depends on the administered construct, including its hydrated dimensions, surface chemistry, protein interactions, and aggregation state. Transmission electron microscopy describes a dry-state core and cannot alone establish renal filterability of the loaded formulation. An instructive comparison involved approximately 100 nm polyethyleneimine-crosslinked assemblies containing multiple carbon dots. Imaging supported hepatobiliary transport and fecal elimination of these assemblies, whereas the individual polyethyleneimine-passivated dots showed a different clearance pattern. This synthetic system demonstrates that incorporating small dots into a larger construct can change biological fate; it does not provide a clearance rule for medicinal-plant-derived dots (Liao et al., 2021, p. 1).

The sucrose-derived doxorubicin study provides a further interpretive caution. Although its title describes renal-clearable carbon dots, the reported biodistribution imaging of dye-loaded material does not, by itself, quantify the fraction of intact drug-loaded dots eliminated in urine. Kidney-associated signal and urinary excretion are distinct endpoints (Azmi et al., 2025, p. 2751). A botanical candidate should therefore undergo direct collection and analysis of excreta if renal clearance is a central development claim.

Chemical transformation and an evidence-based mass balance

Loss of fluorescence should not be equated with carbon-dot degradation. A signal may decrease because of quenching, redistribution, chemical alteration of the fluorophore, or elimination of a label. Conversely, a persistent signal does not establish that the original carrier remains chemically intact. Claims of biodegradation require analytical evidence that identifies the material remaining and

its transformation products. The plant origin of a precursor does not demonstrate degradability of the carbonized product.

As an author-proposed framework, urine and feces should be collected at defined intervals and analyzed for carrier-related material, drug, and identifiable drug metabolites. Recovery should be expressed relative to administered dose, with the analytical method, label stability, and unrecovered fraction stated. Measurements in plasma and tissues should complement excreta data rather than substitute for them. If intact particles cannot be distinguished from soluble products, the result should be described as elimination of measured carrier-related species. Repeated exposure also warrants examination when the intended formulation is administered more than once.

Table 1 translates these considerations into practical questions for botanical carbon-dot development. It is a proposed evaluation framework, not a claim that every reviewed preparation has already satisfied these requirements. Its purpose is to connect material identity and formulation behavior with interpretable exposure and elimination data.

Table 1. Proposed biopharmaceutical evaluation framework for botanical carbon-dot formulations.

Development question	Measurements to consider	Interpretation supported
How much drug is associated?	Validated drug assay, free-drug removal, recovery, and stated mass denominators	Loading efficiency and content are distinguished
Is release formulation-dependent?	Free-drug control, membrane calibration, medium conditions, and drug stability	Carrier release is separated from assay limitations
Does the payload cross a barrier?	Receiver-side drug assay, carrier tracking, barrier integrity, and viability	Transport is distinguished from retention or injury

Development question	Measurements to consider	Interpretation supported
Where do drug and carrier distribute?	Plasma and tissue time courses, matrix calibration, and label stability	Analyte-specific exposure is established
Which species leave the body?	Interval urine and feces collection; drug, metabolite, and carrier-related assays	Elimination is distinguished from organ localization
Is the material chemically transformed?	Orthogonal identification of residual carrier and transformation products	Degradation is distinguished from signal loss

Source: Biopharmaceutical evaluation framework developed by the author, informed by studies of release methods, protein interactions, barrier transport, and biological fate (Arezki et al., 2022, p. 14695; Liao et al., 2021, p. 1; Licciardello et al., 2018, p. 9880; Modi & Anderson, 2013, p. 3076; Seven et al., 2021, p. 3942).

Biocompatibility and Therapeutic Interpretation

A delivery formulation should improve a defined exposure or response without introducing an unacceptable biological effect at the intended dose. This requires comparisons among free drug, unloaded carrier, and the loaded preparation, together with the formulation vehicle when relevant. Drug-equivalent comparisons are essential for evaluating therapeutic effects, while carrier-equivalent comparisons help identify carrier-associated effects. The author proposes stating both dose quantities and the exposure duration for every biological test.

Biocompatibility cannot be transferred from one carbon-dot family to another. In the study of polyethyleneimine-containing assemblies, the larger multiple-dot construct and the individual passivated dots differed in biological behavior and compatibility. That finding concerns the specific materials studied rather than a general safety advantage of carbon dots (Liao et al., 2021, p. 1). Protein adsorption and macrophage responses in a separate surface-charge study further support evaluating the biological interface of the actual formulation (Arezki et al., 2022, p. 14695).

The next experimental step should be chosen according to the intended use. For a local formulation, tissue compatibility and drug retention should be assessed together with the desired local response. For a systemic formulation, plasma and target-tissue exposure should accompany efficacy measurements, with clearance organs included where appropriate. Relevant empty-carrier controls and optical-interference controls remain necessary when the preparation itself is fluorescent or biologically active. An improved response in cultured cells does not establish improved systemic bioavailability, and a favorable animal result does not define suitability for human use.

Development Priorities and Future Perspectives

The most useful development pathway begins with the target and route, then identifies the measurements needed to test the formulation hypothesis. For example, a release design should specify where release is expected, what exposure duration is useful, and how the drug remains chemically active during that period. Barrier experiments should then establish whether the payload reaches the relevant compartment, followed by studies that connect drug and carrier exposure with the intended response. This sequence is proposed as a research strategy rather than evidence that current botanical formulations already meet each requirement.

Reproducibility should be examined at the level of the administered preparation. Independent botanical feedstock lots and synthesis batches should be carried through purification, drug loading, and biological testing. Release and exposure measurements should use a stable dose definition so that batch variation is distinguishable from analytical variation. A highly fluorescent batch is not necessarily the batch with the most useful loading, tissue exposure, or elimination behavior.

Future work would be strengthened by measurements that connect the different stages of delivery in the same formulation. Plasma drug analysis, carrier tracking, target-tissue assessment, and excreta collection can then be interpreted together rather than assembled from experiments using different labels or surface modifications. Where direct identification of the intact carrier is technically limited, the measured species and analytical uncertainty should be reported explicitly. A meaningful development decision depends on knowing which component reached a site and which component left the body.

Conclusion

Botanical carbon dots offer a basis for investigating pharmaceutical delivery, with selected studies demonstrating drug association, condition-dependent release, and biological responses in experimental systems. Their usefulness as carriers depends on the final drug-loaded preparation and on the route-specific delivery problem. Material characterization, loading definitions, and validated analytical methods are therefore part of the biopharmaceutical assessment rather than preliminary descriptive exercises.

The most defensible interpretation links release to barrier transport, drug and carrier exposure, and measured elimination. Cellular uptake, tissue fluorescence, and kidney localization each provide information, but they resolve different questions. Mechanistic studies of other carbon-dot systems help identify useful controls without establishing a universal behavior for botanical formulations. Progress requires studies that maintain the identity of the tested preparation across these stages and connect its biological fate with a reproducible therapeutic objective.

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CHAPTER 3

ANTIMICROBIAL AND ANTIOXIDANT POTENTIAL OF CARBON DOTS DERIVED FROM MEDICINAL AND AROMATIC PLANTS

ASLIHAN CESUR TURGUT[1]

Introduction

The scientific value of converting medicinal and aromatic plants into carbon nanomaterials extends beyond obtaining a fluorescent product from an inexpensive feedstock. Botanical resources contain multiple classes of carbon-containing constituents, and processing can redistribute these constituents into new molecular and particulate forms. Consequently, the relevant research question is whether the transformation produces a reproducible material with a biological function that can be distinguished from the activity of the original extract.

Early investigations established fluorescent carbon-containing species during carbon nanotube purification and subsequently demonstrated strongly luminescent carbon dots following surface passivation. These studies provide historical foundations, but they do not establish the identity or biological

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behavior of every modern biomass-derived product (Sun et al., 2006, p. 7756; Xu et al., 2004, p. 12736). Plant-based preparations therefore require their own chemical and functional evidence.

The present chapter is a critical narrative review of selected experimental studies and foundational methodological papers, rather than an exhaustive systematic review. Its scope includes medicinal and aromatic plant biomass, selected plant-processing residues, isolated botanical compounds used as precursors, and related composite systems where the distinction is scientifically informative. Tea and apple residues are included as contextual examples of biomass valorization. They should not be treated as interchangeable with every medicinal or aromatic plant feedstock.

A central interpretive problem is the coexistence of antioxidant and antimicrobial claims. Radical scavenging may protect a material or biological system from oxidative damage, whereas some antimicrobial preparations act by increasing oxidative injury. These functions need not be mutually exclusive, but each must be demonstrated under defined conditions. This chapter develops a framework in which precursor chemistry, processing, purification, surface structure, biological environment, and formulation are examined together.

Terminology and Material Identity

Carbon dots and carbon quantum dots

Carbon dots (CDs) form a broader family than carbon quantum dots (CQDs). Structural discussions distinguish graphene quantum dots, CQDs, and carbonized polymer dots, with the latter retaining substantial polymer-like features. Nomenclature varies across publications; Zeng and colleagues explicitly emphasized precursor-dependent structural diversity in their proposed classification (Zeng et al., 2021, p. 1). A fluorescent plant-derived

dispersion should therefore not be assigned to a particular subtype solely because it emits visible light.

In this chapter, CD is used where the available evidence supports a carbon-dot designation but does not resolve the subtype. CQD is retained when discussing studies using that terminology, without implying that quantum confinement has been independently demonstrated. This distinction matters because a material dominated by molecular fluorophores or partially carbonized polymers may behave differently from a more extensively carbonized particle.

Botanical origin describes more than one material class

Three frequently conflated systems should be separated. Whole-plant or extract-derived dots arise from the transformation of a chemically mixed botanical feedstock. Phytochemical-derived dots are prepared from an isolated compound, often together with another reagent. Botanical-active composites contain a plant compound added to a dot or polymer system after, or alongside, material preparation. Tea-derived dots loaded with curcumin illustrate the composite category, while curcumin combined with ethylenediamine provides an example of a phytochemical-based mixed precursor system (Miao et al., 2025, p. 1; Wen et al., 2023, p. 1).

Table 1. Material classes used in the evaluation of botanical carbon-dot systems.

Material class	Origin of the carbon-dot fraction	Main attribution question	Representative study
Mixed botanical precursor	Plant tissue or extract transformed during synthesis	Does activity remain in the purified particulate fraction?	Turmeric leaves; (Saravanan et al., 2021, p. 1)

Material class	Origin of the carbon-dot fraction	Main attribution question	Representative study
Isolated phytochemical precursor	Purified plant compound, with or without a co-precursor	What contributions arise from the phytochemical and added reagent?	Curcumin/ethylene diamine; (Miao et al., 2025, p. 1)
Botanical-active composite	Dots combined with an externally supplied plant active	Is the response caused by the dots, cargo, or their combination?	Tea dots/curcumin; (Wen et al., 2023, p. 1)
Dot-containing formulation	Previously prepared dots incorporated into a film or carrier	Does the formulation preserve activity and alter exposure?	Rosemary-dot biopolymer films; (Rodríguez-Varillas et al., 2022, p. 1)

Source: Author's classification based on selected studies (Miao et al., 2025, p. 1; Rodríguez-Varillas et al., 2022, p. 1; Saravanan et al., 2021, p. 1; Wen et al., 2023, p. 1).

Table 1 is an interpretive classification developed for this chapter; it is not a replacement for structural CD nomenclature. Keeping these categories separate prevents an improvement obtained by loading curcumin from being attributed automatically to intrinsic activity of the unloaded dots.

Medicinal and Aromatic Plants as Precursors

Precursor composition and botanical authentication

The feedstock should be defined by species, plant part, collection or cultivation conditions, pretreatment, and extraction procedure. A label such as "plant-based CQDs" gives insufficient information for reproducibility. In the neem study of Gedda and colleagues, leaves were used to generate a fluorescent product evaluated for antioxidant and antimicrobial responses. Mardasi and colleagues compared *Salvia hydrangea* aerial material with plant cell cultures as alternative sources, demonstrating the value of

distinguishing precursor types within a single species (Gedda et al., 2023, p. 1; Mardasi et al., 2025, p. 1).

A useful precursor record would include a taxonomic identification, voucher information where available, moisture content, particle size after milling, and an analytical description of the starting extract. These are proposed reporting elements, not universal quality thresholds. Their purpose is to make it possible to identify whether changes in the final material originate from the biological feedstock or from processing.

Transformation is different from phytochemical preservation

Carbonization can reorganize the constituents of a botanical mixture. An FTIR band consistent with an oxygen-containing functional group does not identify a particular original phenolic compound. Claims that a plant active survives synthesis require direct molecular evidence or suitably designed comparison experiments. Chromatographic separation studies have shown that reaction mixtures can contain freely dissolved fluorophores, particle-associated fluorescent species, and less fluorescent carbon-rich fractions (Hinterberger et al., 2019, p. 8464; Otten et al., 2022, p. 6148).

The following questions provide a practical interpretation sequence: which constituents are present before processing; which soluble products remain after processing; what is associated with the isolated particulate fraction; and which fraction produces the measured activity? Answering them is more informative than describing all detected surface groups as preserved botanical bioactives.

Processing residues and sustainability

Plant residues can provide useful feedstocks, but waste utilization and environmental superiority are different claims. Murru

and colleagues studied dots prepared from tea and grape wastes, while Rodríguez-Varillas and colleagues evaluated rosemary- and apple-pomace-derived dots in biopolymer films (Murru et al., 2020, p. 1; Rodríguez-Varillas et al., 2022, p. 1). These examples establish possible routes for valorization; they do not by themselves quantify the environmental advantage of an entire production process.

For aromatic plants, post-distillation biomass is an attractive candidate for investigation. However, essential-oil extraction changes the feedstock, so results obtained with intact leaves should not be transferred directly to the spent biomass. A proposed sustainability assessment should account for drying energy, extraction inputs, reaction energy, purification water, material yield, and disposal of residual fractions. The preferred process would combine useful performance with a demonstrable reduction in resource burden.

Synthesis, Purification, and Reproducibility

Synthesis routes and their interpretation

Hydrothermal treatment is represented in several botanical studies, including turmeric leaves, rosemary leaves, and *Polygonatum kingianum* or *Gastrodia elata*. Neem-derived dots have also been reported using a sand-bath preparation. These methods differ in precursor handling and reaction environment and should not be described as chemically equivalent (Eskalen et al., 2021, p. 428; Gedda et al., 2023, p. 1; Saravanan et al., 2021, p. 1; Zhang et al., 2025, p. 23845).

For a comparative synthesis study, temperature, time, precursor loading, solvent composition, pH, and co-precursor ratio should be varied with a clearly stated objective. The highest fluorescence intensity need not identify the preparation with the strongest biological activity. Optical signal depends on emissive species and measurement conditions, while antimicrobial or

antioxidant responses depend on the composition and accessibility of reactive sites.

Microwave treatment and thermal routes may offer alternative processing strategies, but their suitability should be assessed using actual yield, energy input, product distribution, and biological performance. Short reaction time alone does not establish process efficiency. A convincing optimization compares purified materials at an equivalent, explicitly defined dose.

Purification as a scientific requirement

Purification determines what material is being tested. Centrifugation or a conventional syringe filter can remove large debris while leaving soluble metabolites and small reaction products in the dispersion. Dialysis, chromatography, or combinations of separation methods may be required, depending on the preparation. Hinterberger and colleagues resolved different fluorescent and particulate fractions chromatographically; Otten and colleagues examined interactions between molecular fluorophores and larger products during purification (Hinterberger et al., 2019, p. 8464; Otten et al., 2022, p. 6148).

The biological importance of purification is illustrated by a *Plantago major* study that examined filtration and dialysis in relation to therapeutic and antimicrobial properties (Altinkaya, 2025, p. 1). The general lesson is methodological: changing the separation procedure changes the tested mixture and may change the interpretation of its activity.

An informative experimental design would retain the crude product, purified retentate, and removed soluble fraction for parallel testing. If activity decreases after purification, this should prompt investigation of the active component rather than an assumption that purification damaged the dots. Conversely, persistent activity in a

purified fraction strengthens attribution, provided that residual contamination has been assessed.

Yield and batch consistency

A reproducible process needs a defined product yield and dose basis. The dry mass of a crude dispersion may contain salts, free metabolites, and other nonparticulate components. Reports should specify whether concentrations refer to crude solids, purified material, or another analytical measure. Independent synthesis batches should be characterized and tested separately; multiple measurements from one batch describe analytical repeatability, not manufacturing reproducibility.

Characterization Relevant to Biological Activity

A combined characterization strategy

Structural and chemical measurements should answer complementary questions. TEM provides information about particle morphology and dry-state dimensions. DLS describes hydrodynamic behavior in a dispersion; its result should not be treated as a second measurement of exactly the same quantity. FTIR supports functional-group analysis, while XPS adds surface-sensitive elemental and chemical-state information. Diffraction and Raman measurements can contribute evidence on carbon organization. Botanical studies using combinations of these methods show why multiple measurements are needed (Mardasi et al., 2025, p. 1; Saravanan et al., 2021, p. 1; Zhang et al., 2025, p. 23845).

The rosemary study reported a mean particle diameter of 16.13 nm, illustrating that materials described as CDs do not invariably fall within a strict sub-10-nm size convention (Eskalen et al., 2021, p. 428). A review should preserve such reported findings instead of forcing all preparations into one size definition.

Optical properties and purity

UV–visible absorption, excitation–emission measurements, fluorescence lifetime, and quantum yield describe optical behavior, but they do not independently establish a unique particle structure. Molecular fluorophores can remain associated with carbon-containing products or contribute to emission from the surrounding solution. Separation coupled with chemical analysis is therefore important when fluorescence is used to track material formation or biological uptake (Otten et al., 2022, p. 6148).

For bioimaging, a proposed control is to compare purified particles with the removed fluorescent fraction. Fluorescence within cells demonstrates uptake of an emissive species; establishing uptake of the intended nanomaterial requires additional evidence. Similarly, strong emission should not be presented as an indicator of high antioxidant or antimicrobial potency.

Characterization in the application medium

Measurements in water are a starting point. A formulation may expose the dots to electrolytes, proteins, surfactants, or polymer networks that alter their dispersion and available surface. The recommended characterization sequence therefore progresses from the purified stock to the biological test medium and finally to the intended formulation. Changes in size distribution, optical response, or surface charge should be considered alongside any change in biological performance.

Antioxidant Potential

Surface chemistry and proposed mechanisms

Antioxidant activity can involve reactions at surface sites and interactions with conjugated structures, but a single mechanism should not be assigned to every botanical dot. Ji and colleagues chemically modified carboxyl and amino groups to examine their

contributions to DPPH radical scavenging. Their results support involvement of these groups in direct or indirect hydrogen-transfer processes. Murru and colleagues considered several possible radical-scavenging pathways in biomass-derived preparations (Ji et al., 2019, p. 1; Murru et al., 2020, p. 1).

These studies support a structure–activity approach. For an uncharacterized botanical preparation, however, the presence of a hydroxyl or amino band is insufficient to quantify its contribution. Chemical modification, fractionation, and suitable reaction measurements provide stronger evidence than a correlation between an FTIR spectrum and an endpoint antioxidant percentage.

Chemical antioxidant assays

DPPH, ABTS, and FRAP evaluate different aspects of reactivity. The DPPH method monitors the response of a stable radical to an antioxidant. The improved ABTS assay uses a preformed radical cation, while FRAP measures ferric reduction under specified assay conditions. These foundational methods were developed independently and their numerical results are not interchangeable (Benzie & Strain, 1996, p. 70; Brand-Williams et al., 1995, p. 25; Re et al., 1999, p. 1231).

For carbon-dot samples, the proposed assay controls include a concentration-matched sample blank without the reporter reagent, a reagent control, and a reference antioxidant. Where appropriate, a second analytical readout should be used to test whether sample absorbance, fluorescence, scattering, or reporter interaction distorts the endpoint. A decrease in optical signal must be distinguished from a chemically verified decrease in the measured radical or oxidant.

Antioxidant percentages should always be linked to concentration, reaction time, solvent, and calculation method. IC50 and EC50 values require an explanation of the fitted response. Reporting a mass-based IC50 for a heterogeneous dot preparation

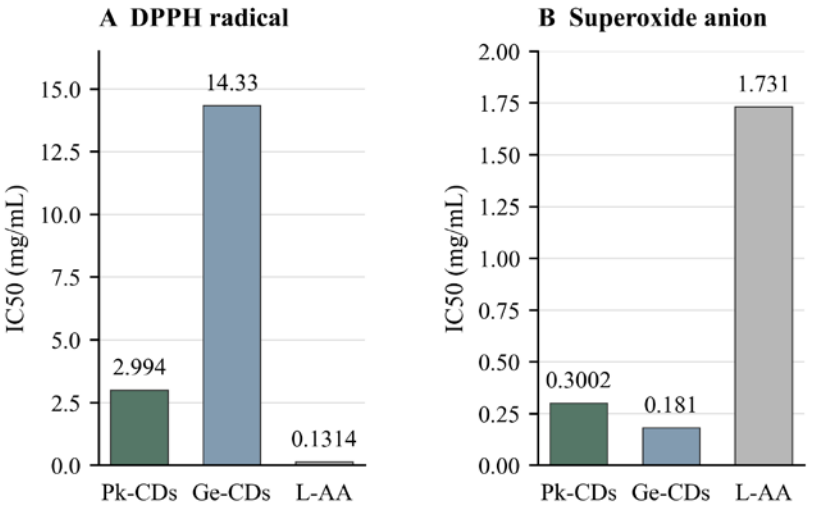
does not establish that it is intrinsically more effective than a molecular antioxidant measured on a molar basis.

Radical-dependent behavior and biological relevance

Different radicals can produce different rankings. In the experimental results of Zhang and colleagues, *P. kingianum*-derived dots showed a DPPH IC50 of 2.994 mg/mL, compared with 14.33 mg/mL for *G. elata*-derived dots. For superoxide scavenging, the respective reported IC50 values were 0.30 and 0.18 mg/mL. These outcomes favor different materials depending on the endpoint and argue against a single ranking of overall antioxidant superiority (Zhang et al., 2025, p. 23845).

Figure 1 makes the assay dependence of these comparisons visible and includes the molecular reference antioxidant used in the source study. The relative positions of the two dot preparations differ between the DPPH and superoxide assays, so the result should be interpreted within each assay rather than as a single ranking of biological antioxidant performance.

Figure 1. Assay-dependent antioxidant IC50 values for two botanical carbon-dot preparations and a reference antioxidant.



Source: Original plot prepared by the author using numerical values from the source study's Table 4 (Zhang et al., 2025, p. 23845). Pk-CDs: Polygonatum kingianum dots; Ge-CDs: Gastrodia elata dots; L-AA: L-ascorbic acid. Lower IC50 indicates greater activity within an assay. Panels use different y-axis scales; uncertainty estimates are not plotted.

Moving to a biological model requires evidence beyond a cell-free reaction. The same study examined oxidative-stress-associated markers in a D-galactose mouse model. Such findings extend the evidence within that particular model but do not establish clinical efficacy or safety for other exposure routes (Zhang et al., 2025, p. 23845).

For a proposed skin-related evaluation, chemically verified scavenging should be followed by a relevant cell model, a defined oxidative challenge, and measurements of both viability and oxidative damage. A lower ROS-probe signal is more persuasive when supported by an independent endpoint and when the material

has been tested for probe interference. Protection of cells, prevention of lipid oxidation, and stabilization of a cosmetic formulation are distinct outcomes and should be described separately.

Antimicrobial Potential

Membrane interactions and oxidative injury

Antimicrobial mechanisms may include surface association, membrane damage, and oxidative injury. Turmeric-leaf dots were reported to produce bactericidal responses involving ROS. Quaternized curcumin-derived dots were investigated for enhanced adherence to bacterial membranes, illustrating how an added surface functionality can influence the response (Saravanan et al., 2021, p. 1; Wu et al., 2022, p. 1).

These findings should not be generalized to every plant-derived dot. A proposed mechanistic evaluation combines viable counts with microscopy, membrane-permeability measurements, and tests of the suspected oxidative pathway. If ROS is proposed as the main cause of death, the timing and effect of pathway interventions should support causation. Observing a higher ROS-probe signal and fewer viable bacteria in parallel establishes an association but does not by itself identify the full killing mechanism.

Bacterial spectrum and resistant organisms

Different organisms and strains can respond differently to one preparation. Gram-positive and Gram-negative categories are useful descriptions, but they are insufficient predictors of susceptibility. Miao and colleagues studied curcumin/ethylenediamine-derived dots against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, including methicillin-resistant *S. aureus*. Their analysis included MIC determination, staining, morphological observations, and ROS-related measurements (Miao et al., 2025, p. 1).

Activity against a resistant strain establishes a relevant experimental result for that organism and test condition. It does not demonstrate that resistance cannot develop. A proposed resistance evaluation would examine multiple strains and repeated exposure, with appropriate comparators and clearly defined recovery conditions. Claims such as “resistance-free” should be limited to the experimental evidence actually obtained.

Antifungal activity and biofilms

Antifungal activity requires separate evaluation because fungal targets and growth conditions differ from bacterial ones. Neem-derived dots have been tested against bacterial and fungal strains using diffusion-based assays (Gedda et al., 2023, p. 1). Such results support further investigation, but a diffusion zone alone does not identify fungicidal activity or establish effectiveness against an established fungal biofilm.

For biofilm studies, prevention of attachment, reduction in total biomass, and killing within a mature biofilm should be treated as different endpoints. The proposed assessment combines a biomass measurement with viable recovery and, where useful, microscopy. A material may reduce matrix-associated staining without producing a corresponding decline in viable organisms; this possibility should be investigated rather than described automatically as eradication.

Light-dependent antimicrobial responses

Carbon-dot systems can also function as components of light-activated treatments. Yan and colleagues developed carbon-dot–curcumin nanocomposites with photodynamic and photothermal antibacterial properties. This study concerns an engineered composite and should not be used as evidence that every unloaded botanical dot produces the same light response (Yan et al., 2021, p. 6703).

A proposed factorial design includes untreated, light-only, material-only, and material-plus-light groups. Composite studies additionally require dots-only and active-only comparisons. Wavelength, irradiance, exposure time, delivered energy, and temperature should be reported. Separating light-driven chemistry from heating is particularly important when both photodynamic and photothermal effects are proposed.

Antimicrobial assay quality

MIC measurements require standardized growth conditions and a clear endpoint. EUCAST provides current recommendations for broth microdilution of aerobic and facultative anaerobic bacteria. These recommendations offer a methodological reference for controlling media, inoculum, and incubation, but clinical antibiotic breakpoints should not be transferred to an experimental carbon-dot preparation without validation (European Committee on Antimicrobial Susceptibility Testing, 2026, p. 1).

Carbon-dot assays also need controls for intrinsic color, turbidity, reporter interactions, and aggregation. MIC describes inhibition under the specified conditions; a bactericidal claim requires appropriate viable recovery evidence. Diffusion zones can be useful for screening, but the transport of the material through agar affects interpretation. Results from diffusion, broth inhibition, and time-kill experiments should therefore be reported with their own endpoint definitions.

Selected Experimental Studies and Comparative Interpretation

The studies in Table 2 illustrate distinct feedstocks and evidence types. They are selected examples, not a quantitative meta-analysis. Their concentrations, assays, organisms, and purification procedures differ, preventing a defensible pooled ranking.

Table 2. Selected botanical carbon-dot studies and the scope of their findings.

Precursor or system	Preparation and material evidence	Biological finding	Interpretive scope and reference
Curcuma longa leaves	Hydrothermal preparation; mean particle size 2.6 nm	At 0.25 mg/mL, complete killing of *E. coli* and *S. aureus* was reported after 8 h	A concentration- and exposure-specific bactericidal result; (Saravanan et al., 2021, p. 1)
Rosemary leaves	Hydrothermal preparation; mean diameter 16.13 nm	Microbial testing and a PVA-containing fruit-storage demonstration	Storage observations are qualitative and formulation-dependent; (Eskalen et al., 2021, p. 428)
Azadirachta indica leaves	Sand-bath preparation; TEM diameter approximately 3.5 nm	Radical-scavenging and diffusion-based antibacterial / antifungal responses	Read with dose, assay, and fraction-purity information; (Gedda et al., 2023, p. 1)
Tea and grape wastes	Biomass-derived dots characterized for chemical and optical properties	Antioxidant testing in aqueous media; selected preparations tested in oil	Matrix-dependent performance; (Murru et al., 2020, p. 1)
Rosemary and apple-pomace dots in biopolymer films	Dots incorporated at 0.25–1% by mass	Antioxidant-film evaluation, including edible and cosmetic oil samples	Proof of concept for a film system; (Rodríguez-Varillas et al., 2022, p. 1)
Green-tea dots loaded with curcumin	Hydrothermal dots combined with an external botanical active	Antioxidant and antibacterial nanocomposite evaluation	Composite response requires comparison with both components; (Wen et al., 2023, p. 1)
Salvia hydrangea aerial parts and cell cultures	Separate hydrothermal precursor routes	Antimicrobial, antioxidant, and cytotoxicity evaluations	Supports investigation of precursor-dependent properties; (Mardasi et al., 2025, p. 1)

Precursor or system	Preparation and material evidence	Biological finding	Interpretive scope and reference
Plantago major	Hydrothermal preparation with purification comparisons	Evaluation of antibacterial, antioxidant, and other biological responses	Purification is part of material definition; (Altinkaya, 2025, p. 1)
Polygonatum kingianum and *Gastrodia elata*	Separate hydrothermal products; mean sizes 6.2 and 6.8 nm	DPPH, superoxide, antibacterial, and an animal oxidative-stress model	Rankings depend on assay; (Zhang et al., 2025, p. 23845)

Source: Author's compilation of selected studies (Altinkaya, 2025, p. 1; Eskalen et al., 2021, p. 428; Gedda et al., 2023, p. 1; Mardasi et al., 2025, p. 1; Murru et al., 2020, p. 1; Rodríguez-Varillas et al., 2022, p. 1; Saravanan et al., 2021, p. 1; Wen et al., 2023, p. 1; Zhang et al., 2025, p. 23845).

A botanical label does not explain these differences. The relevant comparison begins with material class and fraction purity, then proceeds to dose basis, medium, and endpoint. Even studies sharing the same plant name may involve different extracts, tissue states, added reagents, or purification procedures.

Three attribution questions emerge from this comparison. First, can activity be assigned to the purified carbon-dot fraction? Second, is a measured improvement retained when components are compared at equivalent doses? Third, does the response persist in the intended application environment? These questions provide a more useful foundation for research planning than a table of the largest reported inhibition zones or scavenging percentages.

Linking Antioxidant and Antimicrobial Functions

A multifunctional material must be evaluated as a system. A preparation that consumes radicals in a chemical assay may still alter microbial survival through membrane effects or through oxidative processes under another exposure condition. Likewise, attaching an antioxidant cargo to a photoactive carrier may change both

functions. This chapter proposes evaluating redox behavior and antimicrobial activity in the same relevant medium, dose range, and illumination conditions whenever a dual function is claimed.

The main translational question is selectivity. For an infection-related application, microbial injury should be assessed together with compatibility toward relevant host cells. For packaging, the goal may instead be to reduce microbial contamination while slowing product oxidation. For cosmetics, preventing lipid oxidation and preserving a formulation are separate from any claimed effect on skin biology.

Table 3. Proposed controls for attributing biological activity in botanical carbon-dot systems.

Evaluation question	Comparison or control	Interpretation enabled
Do soluble reaction products contribute?	Crude dispersion, purified dots, and removed fraction	Distinguishes particulate activity from mixture activity
Does the original extract explain the response?	Starting extract at a clearly justified equivalent basis	Tests changes introduced by synthesis
Is a loaded active responsible?	Dots, active, physical mixture, and engineered composite	Separates carrier, cargo, and combination effects
Does light change the mechanism?	Light-only, material-only, and combined exposure	Resolves dark and photoactivated behavior
Does the material distort the assay?	Sample blanks and an independent biological or chemical endpoint	Tests optical or reporter interference
Is the effect selective?	Microbial viability and relevant host-cell responses	Relates efficacy to compatibility

Evaluation question	Comparison or control	Interpretation enabled
Is the product reproducible?	Independently synthesized and tested batches	Separates batch variation from measurement repeatability

Source: Evaluation framework developed by the author, informed by purification, antimicrobial-method, and composite studies (European Committee on Antimicrobial Susceptibility Testing, 2026, p. 1; Hinterberger et al., 2019, p. 8464; Otten et al., 2022, p. 6148; Wen et al., 2023, p. 1).

Table 3 is a proposed evaluation framework, not a claim that all cited studies performed every comparison. Its value lies in assigning a specific alternative explanation to each control. A control becomes scientifically useful when it resolves an attribution problem rather than merely increasing the number of experimental groups.

Application Potential

Antimicrobial materials and wound applications

Potential applications include antimicrobial carriers, wound-associated materials, and combined imaging/treatment systems. Quaternized curcumin-derived dots were investigated for infected wound healing, providing an example of an engineered material designed around a specific biological problem (Wu et al., 2022, p. 1). Translation requires attention to the material used in that study; similar behavior cannot be presumed for a different botanical precursor or surface treatment.

For a proposed wound formulation, testing should distinguish release of the active material from immobilized contact effects and should consider fluid composition and exposure duration. Host-cell compatibility, microbial killing, and tissue-related outcomes need separate measurements. Fluorescent tracking may support distribution studies but does not substitute for establishing clearance or biological safety.

Packaging and product protection

Dot-containing films offer an application in which the final matrix is central to performance. Rodríguez-Varillas and colleagues investigated antioxidant biopolymer films containing rosemary or apple-pomace dots, including proof-of-concept tests with edible and cosmetic oils. This evidence supports further material development; it does not establish suitability for every packaging configuration or contact condition (Rodríguez-Varillas et al., 2022, p. 1).

A proposed packaging study should assess oxidative stability, microbial response where relevant, migration, barrier properties, and mechanical behavior over the intended storage period. Protection observed during a short experiment should not be presented as a validated commercial shelf-life extension.

Cosmetic and dermocosmetic development

For cosmetic applications, the immediate research opportunities are protection of formulation ingredients against oxidation and investigation of skin-related effects in suitable models. The oil-protection example above concerns a material–product interaction, not proof of an anti-aging effect on human skin. This distinction should remain explicit when discussing botanical dots as candidate cosmetic ingredients.

Emulsions and gels introduce additional questions: where the dots reside, whether they aggregate, how they interact with other ingredients, and whether activity changes during storage. A proposed development pathway includes physicochemical stability, photostability, compatibility with the formulation, and biological evaluation relevant to the intended exposure. Antimicrobial activity in a laboratory culture does not establish preservative performance in a finished product. Claims concerning preservation, skin benefits, and antimicrobial treatment require different evidence.

Biocompatibility, Safety, and Environmental Evaluation

Botanical origin does not determine safety. The exposure includes the final particle structure, surface chemistry, residual soluble components, and formulation. Gedda and colleagues reported concentration-dependent compatibility in L929 fibroblasts, while Mardasi and colleagues included cytotoxicity evaluation in their comparison of *Salvia hydrangea*-derived preparations. Such findings are tied to the tested model and conditions (Gedda et al., 2023, p. 1; Mardasi et al., 2025, p. 1).

A proposed safety strategy begins with a well-defined purified material and application-relevant exposure. Cell viability should be interpreted alongside membrane integrity and other appropriate biological endpoints, with interference controls for the chosen readouts. Repeated exposure, light-dependent responses, and the effect of formulation components may require additional assessment. Lack of an adverse response in one assay should not be converted into a universal claim of nontoxicity.

Environmental evaluation should address the final material and the production process separately. An environmentally preferable feedstock can still produce a dispersion that requires assessment of persistence, transformation, or effects on non-target organisms. Conversely, immobilization in a matrix may change exposure but requires evidence on release. Regulatory and product-development decisions should be based on the defined material, intended use, and applicable requirements rather than a general “natural” designation.

Research Priorities and Future Perspectives

The proposed priorities are directed toward resolving uncertainty that limits meaningful comparison. The first is attribution: determine whether the active component is a particulate material, an associated molecule, a soluble reaction product, or a

composite. The second is reproducibility: identify the precursor and manufacturing variables responsible for variation among batches. The third is validation in the intended medium: show that activity survives incorporation into the target formulation or matrix.

Research on aromatic-plant residues would benefit from direct comparisons of intact biomass and processing residues, using a consistent synthesis and purification scheme. Phytochemical fingerprinting before synthesis could then be connected to surface chemistry and biological response after synthesis. This approach would test whether precursor differences have functional consequences without assuming preservation of specific molecules.

Mechanistic work should also examine the boundaries of multifunctionality. Rather than measuring antioxidant activity and bacterial inhibition in unrelated conditions, future studies could map both responses across the same dose, pH, and illumination ranges. Such experiments would identify conditions in which the material protects the target product or host while inhibiting microorganisms.

Finally, scale-up should be judged by yield, reproducibility, purification demand, energy use, and final performance. A successful laboratory synthesis is a starting point. An application-ready process needs acceptance criteria linked to the intended function, with clear limits for variability and impurities. These recommendations constitute a research framework; they are not evidence that current botanical carbon-dot products have already satisfied those requirements.

Conclusion

Medicinal and aromatic plants provide diverse precursors for carbon-dot systems whose antioxidant and antimicrobial responses depend on material composition and exposure conditions. Selected studies demonstrate activity in chemical assays, microbial cultures, films, and defined biological models. These findings support further

development while requiring careful distinction among purified dots, soluble reaction products, and composites containing additional active compounds.

The central requirement is attribution: the measured effect should be connected to a characterized fraction and then confirmed in the intended medium or formulation. Redox behavior and microbial responses should be evaluated together when multifunctionality is claimed, with controls that address optical interference, illumination, component contributions, and host-cell compatibility. Such evidence is more informative than botanical origin or fluorescence alone. Progress toward wound materials, protective films, and cosmetic formulations will depend on reproducible preparation and application-specific validation of the biological function.

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CHAPTER 4

AZOXYSTROBIN EFFECTS ON SOIL DEHYDROGENASE ACTIVITY: EVIDENCE AND ENVIRONMENTAL CONTEXT

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Introduction

Azoxystrobin is a broad-spectrum systemic fungicide in the strobilurin group, used to control fungal diseases in crops (Bartlett et al., 2002:649-662). Although it is commonly applied to foliage, seed, or crop furrows, some of the applied compound can reach soil. Its effects below ground therefore matter alongside its intended activity against plant pathogens (Adetutu et al., 2008: 1777–1790). The extent and duration of soil exposure can vary with conditions: experiments in two rice-growing soils found that azoxystrobin persistence differed between soil types and moisture regimes, and that compost amendment had different effects in the two soils (Singh & Singh, 2010: 676–681). These findings make soil conditions relevant when interpreting reported biological responses.

Soil microorganisms contribute to processes that sustain soil fertility, including organic matter decomposition and nutrient

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cycling (Aka Sağlıker & Cenkseven, 2016:649-655; Boga et al., 2017:1052-1060; Cenkseven, 2026:178-188). Soil enzymes provide measurable indicators of some of these biological processes and can respond to environmental disturbance and management (Utobo & Tewari, 2015:148-149). This broader context is relevant to pesticide exposure because changes in microbial activity or enzyme responses may signal effects on soil functions (Koçak & Cenkseven, 2021:1-2), although individual enzyme results should not be treated as direct evidence of changes in overall soil health (Yasir et al., 2025:11-13).

Dehydrogenase activity is commonly used as an indicator of overall microbial respiratory activity in soil. Because dehydrogenase is associated with cellular metabolism, its measured activity can help characterize microbial responses to disturbance (Neemisha & Sharma, 2022:173-188). However, enzyme assays can differ in their reaction conditions and measurement procedures, which limits direct comparison among studies unless methods and units are considered (Utobo & Tewari, 2015:148-149). Dehydrogenase is therefore useful as a response indicator, but its results need to be interpreted alongside experimental design, soil properties, exposure conditions, and other microbial or biochemical measurements.

Studies of azoxystrobin show why this context matters. Adetutu et al. (2008: 1777–1790) reported changes in fungal community responses under some incubation conditions, while bacterial diversity was unaffected in their experiment. In a separate Spodosol study, azoxystrobin exposure was associated with changes in several enzyme activities, carbon-source utilization, and bacterial community structure; the measured enzymes did not include dehydrogenase (Wang et al., 2020:3-7). These studies point to biological responses that may be relevant to soil functioning, but they do not establish a uniform dehydrogenase response across soils or experimental settings. The broader pesticide literature also links changes in microbial communities and enzyme activity with nutrient

cycling and soil fertility, while recognizing that outcomes depend on the pesticide and soil context (Yasir et al., 2025:11-13).

This review examines the effects of azoxystrobin on soil dehydrogenase activity and considers how those findings relate to the fungicide's environmental fate, soil properties, microbial communities, and other enzyme responses. It distinguishes studies that directly measured dehydrogenase from those that provide supporting evidence on azoxystrobin persistence or other soil biological endpoints. By considering dose, exposure duration, soil conditions, and measurement methods, the review aims to clarify what the available studies show and where the evidence remains limited.

Literature Identification and Evaluation

Sources and search terms

The literature considered in this chapter was identified using the Web of Science Core Collection and supplemented with relevant full-text articles and review papers examining azoxystrobin fate, soil microbial responses, and soil enzyme interpretation

The concepts used to organize the literature included *azoxystrobin*, *soil*, *dehydrogenase*, *soil enzyme activity*, *soil microbial activity*, *soil microbial community*, *pesticide degradation*, and *soil fertility*. These terms describe the subject areas covered by the supplied records and articles; they should not be interpreted as a fully documented database search string. The search date, exact Boolean operators, and any filters applied to the Web of Science search were not available in the records provided.

Study selection and relevance

Studies were considered directly relevant when they investigated azoxystrobin exposure in soil and reported dehydrogenase activity. Studies addressing azoxystrobin

degradation or persistence, other soil enzyme activities, microbial activity, or microbial community structure were retained as supporting evidence when they helped explain soil exposure or provide context for biological responses.

Reviews and meta-analyses on soil enzymes were used to inform the interpretation of dehydrogenase activity and methodological differences. They were not treated as primary experiments testing azoxystrobin. Studies conducted in specialized systems, such as biobed substrates, or involving amendments and microbial inoculation were considered separately because their conditions do not directly represent an ordinary agricultural-soil exposure experiment.

Evaluation and limitations

Findings were compared with attention to soil type and properties, azoxystrobin dose, formulation, exposure and sampling duration, amendments, experimental setting, and dehydrogenase assay method. Numerical results were interpreted in light of the units and comparison groups reported by each study. Where an article reported a result only in a figure, that value should be identified as figure-derived and distinguished from values reported in tables or the main text.

Direct comparison among studies is limited by differences in experimental design, measurement protocols, reporting units, and the availability of treatment and control means, measures of variability, and sample sizes. Repeated sampling times from the same experiment and multiple dose groups sharing a control are statistically dependent; they should not be treated as independent observations in a pooled analysis without an appropriate method to account for that dependence. For these reasons, the present chapter uses a narrative synthesis and does not calculate a single pooled effect estimate.

Soil Dehydrogenase Activity

Biochemical basis and significance

Dehydrogenases are oxidoreductase enzymes involved in the transfer of hydrogen and electrons from organic substrates to acceptors during oxidation shown in Figure 1. In soil, dehydrogenase activity (DHA) is commonly used as an indicator of microbial oxidative activity and of transformations associated with organic matter. The principal reason for its use is that soil DHA is generally associated with intact, viable microbial cells. Unlike many extracellular enzymes that can persist after release into the soil matrix, dehydrogenase activity is rapidly lost following cell death. This makes DHA a useful measure of a biologically active component of soil processes (Nannipieri et al., 2018:12-13; Utobo & Tewari, 2015:148-149).

DHA is therefore often described as a sensitive biological indicator of soil condition. It can respond to changes caused by environmental disturbance or management, and has been applied in studies of natural, agricultural, reclaimed, and mine-affected soils (Kumar et al., 2013:899-902; Utobo & Tewari, 2015: 148-149). Its value lies in the fact that it provides information about microbial oxidative processes that may not be apparent from soil chemical measurements alone. However, DHA should be interpreted as one indicator of soil biological functioning, rather than as a complete measure of soil health or microbial abundance.

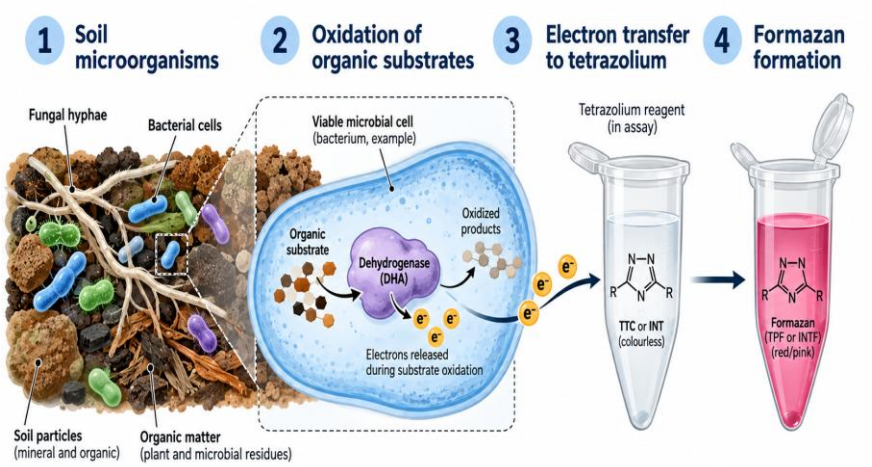
A distinction is needed between *microbial activity*, *microbial biomass*, and *dehydrogenase activity*. Although DHA is often used as a proxy for microbial activity, the measured enzyme response does not necessarily track every aspect of microbial functioning. Nannipieri et al. (2018:12-13) note that DHA does not always correlate closely with other measures such as oxygen consumption. Consequently, DHA should not be treated as interchangeable with

respiration, total microbial biomass, or the activity of the entire soil microbial community. It reflects a particular, assay-defined aspect of microbial oxidative activity.

Principles of measurement

Soil DHA is commonly assessed by adding a tetrazolium compound that can act as an artificial electron acceptor (Figure 1). Microbial reduction of the compound produces a coloured formazan product, which is extracted and quantified, typically by spectrophotometry. The two substrates are triphenyltetrazolium chloride (TTC) and iodonitrotetrazolium chloride (INT). The resulting products are commonly reported as triphenyl formazan (TPF) or iodonitrotetrazolium formazan (INTF), respectively (Kumar et al., 2013:899–902; Utobo & Tewari, 2015:148-149).

Figure 1. Soil dehydrogenase activity



The assay is practical and has been widely adopted, but it does not directly measure an undisturbed, in situ rate of soil metabolism. Instead, it measures the formation of formazan under the specific conditions established by the assay. Substrate identity and concentration, soil mass and moisture, incubation duration and

temperature, pre-incubation, aeration, extraction, and the analytical procedure can all influence the result (Kumar et al., 2013: 899–902; Nannipieri et al., 2018:12-13). Values obtained using different protocols are therefore not automatically comparable, even when both studies describe their endpoint as "dehydrogenase activity."

The reporting unit also matters. Studies may express activity per unit of dry soil and time, but use different mass units or formazan products. A value reported as TPF cannot be assumed to be directly equivalent to a value reported as INTF, and results reported per gram, kilogram, or another soil basis require appropriate conversion before comparison. Such conversions do not correct for differences in assay chemistry or incubation conditions. For quantitative synthesis, the assay method, substrate, incubation conditions, soil basis, time unit, and reported variability should be recorded alongside the numerical result.

Factors influencing measured activity

The observed DHA response is shaped by both soil conditions and laboratory protocol. Moisture and aeration affect the environment in which microbial oxidation occurs; temperature, incubation duration, and pre-incubation can also change formazan production. Kumar et al. (2013:899–902) emphasize these conditions as important sources of variation and discuss the use of both TTC and INT assays. Utobo and Tewari (2015: 148-149) likewise describe DHA as a responsive indicator whose interpretation depends on the biological and environmental context of the soil.

The soil itself is not a uniform assay medium. Differences in soil type and condition can influence microbial communities and the processes being measured. The review by Kumar et al. (2013: 899–902), for example, compares DHA observations across natural, managed, reclaimed, and mine-affected settings. Those comparisons

illustrate why an activity value should be read in the context of the sampled soil and its history, rather than interpreted against a single universal reference range.

Evidence from a meta-analysis of heavy-metal effects further illustrates the sensitivity of soil enzyme responses to the contaminant and soil context. Aponte et al. (2020:3-9) found that enzyme responses differed among enzyme types and were associated with factors including contaminant characteristics and soil properties; DHA was among the enzyme activities considered. This evidence concerns heavy-metal contamination, not azoxystrobin. It is relevant here as an example of why results for one stressor cannot simply be generalized to another, and why soil and study characteristics should be considered when interpreting or synthesizing enzyme data.

Interpretation and limitations

DHA is useful because it is linked to viable-cell oxidative processes and can respond to soil disturbance. Its interpretation nevertheless requires several qualifications. First, a change in DHA indicates a change in the assay-measured activity; by itself, it does not identify the ecological mechanism responsible. Second, a higher value should not automatically be interpreted as an improvement in overall soil quality, nor should a lower value alone be taken as proof of broad microbial damage. The direction and meaning of a response depend on the soil, the experimental conditions, the timing of measurement, and the other endpoints considered (Nannipieri et al., 2018:12-13; Utobo & Tewari, 2015:148-149).

Third, comparisons across studies require protocol transparency. Nannipieri et al. (2018:12-13) caution that methodological differences and incomplete descriptions can undermine the interpretation of soil enzyme data and their use in meta-analysis. For DHA, comparisons are especially difficult when studies differ in tetrazolium substrate, incubation conditions, units,

sampling time, or the way results are normalized. These differences should be evaluated before effect estimates are combined.

For a review of azoxystrobin and soil dehydrogenase, DHA is best treated as a biologically informative but method-dependent endpoint. A study's result should be interpreted together with its soil characteristics, exposure conditions, sampling time, assay protocol, and other microbial or enzymatic measurements. This framing allows DHA to contribute evidence about microbial oxidative activity without overstating what a single assay can establish about the wider soil ecosystem.

Properties and Environmental Fate of Azoxystrobin

Mode of action and uses

Azoxystrobin is a synthetic fungicide belonging to the strobilurin group. Strobilurins were developed from naturally occurring fungicidal compounds and became an important class of agricultural fungicides. In their review, Bartlett et al. (2002: 649–662) describe azoxystrobin among the commercially developed strobilurin fungicides and discuss its use across a range of crops and fungal disease-management systems.

Azoxystrobin acts by inhibiting mitochondrial respiration in susceptible fungi. It binds at the quinol oxidation (Qo) site of the cytochrome bc_1 complex in the mitochondrial electron-transport chain. This interferes with electron transfer from cytochrome *b* to cytochrome c_1 and disrupts energy production, including ATP generation (Bartlett et al., 2002: 649–662). This biochemical mode of action underlies its fungicidal activity against plant-pathogenic fungi.

Azoxystrobin has been marketed for preventative and curative disease-control purposes (Edwards et al., 2016:450-457). Its application in agricultural production can involve different delivery

methods, depending on the crop and product use. For example, the Midwestern United States field study by Edwards et al. (2016:450-457) examined a formulation containing azoxystrobin and propiconazole applied to corn by air and to soybean by ground equipment. Because the study evaluated a mixture of active ingredients, its field observations describe the behavior of that formulation and application system; they should not be interpreted as evidence that every observed effect was caused by azoxystrobin alone.

Routes of entry into soil

Azoxystrobin can reach soil during crop spraying when part of the applied spray passes through the crop canopy and deposits at the soil surface. In the field study by Edwards et al. (2016:450-457), canopy penetration to soil level was observed following both aerial and ground applications. The extent of deposition will depend on application conditions and crop canopy characteristics; the study therefore supports canopy penetration as a direct route of soil exposure under the conditions examined.

A second potential route is spray drift beyond the treated crop area. Edwards et al. (2016:450-457) detected drift associated with aerial application, whereas drift was not associated with the ground application in their study. Drift can move fungicide away from the intended application zone, although the study's drift measurements should not be taken as a direct measure of azoxystrobin deposition in every neighboring soil.

Effects of Azoxystrobin on Soil Dehydrogenase Activity

Direction and magnitude of the response

The 13 studies reviewed here do not show one consistent direction or magnitude of change in soil dehydrogenase activity (DHA) following azoxystrobin exposure. Several report inhibition,

often most clearly at early sampling times or higher concentrations. Others report no statistically significant effect, or increases under particular field, soil-amendment, or microbial-inoculation conditions. Differences in soil properties, exposure design, formulation, dose, sampling time, and assay procedure need to be considered before comparing the results.

One clear early response was reported by Bending et al. (2007:84-87). They applied azoxystrobin at 5 mg kg^{-1} to two soils with contrasting management histories. In Long Close soil, DHA was 89.4% of the unamended control at 0.25 months and 86.3% at one month; both reductions were reported as significant ($p < 0.05$). At two months, activity had returned to 97.7% of the control, and at three months it was 113.7%. In the higher-organic-matter Hunts Mill soil, no significant DHA effect was reported. The authors' comparison is particularly useful because the soils had similar pH (6.5) but differed in total organic carbon (1.18% versus 1.62%) and microbial biomass (139.4 versus 622.6 mg C kg^{-1} dry soil). The study therefore documents a soil-dependent response, rather than a uniform effect of the 5 mg kg^{-1} treatment.

Sopeña and Bending (2013:1527-1529) also found lower DHA following azoxystrobin application. Their laboratory experiment used 25 mg kg^{-1} and compared unamended soil with soils containing 0.1%, 1%, or 2% biochar. In unamended soil, DHA was approximately 51%, 58%, and 30% lower in treated than untreated samples at one, three, and five months, respectively. In biochar-amended soils, the reported one-month decreases relative to the matching biochar-only treatments were 28% at 0.1% biochar, 34% at 1%, and 40% at 2%. Thus, biochar increased the underlying DHA but did not remove the azoxystrobin-associated reduction. These comparisons are based on the authors' reported results and Figure 2; the biochar-treated groups must be compared with their corresponding biochar-only controls, not with unamended soil.

Howell et al. (2014:197-198) reported a concentration-dependent decrease and subsequent recovery in DHA in a four-month laboratory experiment. After one month, activity ranged from 75% of the control at 1 mg kg^{-1} to 45% at 25 mg kg^{-1}. These levels persisted to the two-month sampling. By month three, activity was 102.9% and 103.6% of control in the 1 and 5 mg kg^{-1} treatments, and 88.4% and 98.8% in the 10 and 25 mg kg^{-1} treatments, respectively. At month four, DHA in the 10 and 25 mg kg^{-1} treatments had risen to 137.4% and 133.2% of control, whereas values in the 1 and 5 mg kg^{-1} treatments were 87.1% and 97.8%. The authors interpreted the time course as evidence of recovery in DHA, even though azoxystrobin residues were still present in the soil. The late values above the control should not be interpreted as proof that azoxystrobin generally stimulates DHA: the response followed a pronounced early decline and could reflect biological recovery or other changes during incubation.

Several studies with short laboratory incubation periods also found inhibition. Wang et al. (2015:3336–3343) tested 0.1, 1, and 10 mg kg^{-1} in brown soil and measured microbial and enzyme outcomes on days 7, 14, 21, and 28. The article reports reductions in DHA, soil respiration, and viable microbial numbers, and describes DHA as the most sensitive of the four enzyme activities assessed. Dehydrogenase activity was significantly reduced ($p < 0.05$) across all sampling dates and concentrations, although differences between the 1.0 and 10.0 mg kg^{-1} treatments were not significant on days 14 and 21. By day 28, DHA had decreased by 55%, 72%, and 85% relative to the control at 0.1, 1.0, and 10.0 mg kg^{-1}, respectively. In Cambisol, Wang et al. (2018:2777–2780) tested 0.1, 1, and 10 mg kg^{-1} and measured activity on days 7, 14, 21, and 28. DHA was significantly lower in azoxystrobin-treated soils than in the control except on day 7. By day 28, activity was 19.1%, 49.8%, and 69.4% lower than control at 0.1, 1, and 10 mg kg^{-1}, respectively. Negative

correlations between concentration and DHA were reported on days 14, 21, and 28 ($r = -0.87$, -0.77, and -0.80, respectively).

The results of Baćmaga et al. (2015:1-15) were more modest. In a sandy-loam soil, the authors tested 0.075, 2.25, 11.25, and 22.50 mg kg^{-1} and assessed enzymes after 30, 60, and 90 days. At the highest dose, mean dehydrogenase activity was reported to be 7.04% lower. Dehydrogenases were the most resistant of the enzymes assessed in that experiment, and the lowest DHA was observed on day 30. These findings differ in magnitude from the larger response reported by Wang et al. (2018:2777–2780); they should not be treated as interchangeable estimates because the soils, experimental procedures, and dose series differed.

Dose, formulation, and sampling time

The data suggest that higher concentrations can produce larger decreases in DHA, but the pattern is not identical across studies. Howell et al. (2014:197-198) found a stronger initial reduction at higher concentrations, while Wang et al. (2018:2777–2780) reported progressively greater day-28 reductions across 0.1, 1, and 10 mg kg^{-1}. Baćmaga et al. (2015:1-15), by contrast, reported only a 7.04% decrease in mean dehydrogenase activity at 22.50 mg kg^{-1}. These differences caution against treating nominal concentration as the sole determinant of response. Soil type, fungicide preparation, moisture, incubation conditions, and assay method also differ.

The 2022 Quadris® experiment should be distinguished from studies applying azoxystrobin as a technical active ingredient. Boteva et al. (2022:382-392) used Quadris® in loamy-sand soil at azoxystrobin concentrations of 2.90, 14.65, and 35.00 mg kg^{-1} dry soil. Enzyme activity was measured on days 1, 30, 60, 90, and 120. The reported overall DHA means across the sampling period were 20.11 ± 9.2 µg g^{-1} dry soil h^{-1} in the untreated control, 13.51 ± 4.2 at

2.90 mg kg^{-1}, 12.25 ± 4.3 at 14.65 mg kg^{-1}, and 11.36 ± 4.4 at 35.00 mg kg^{-1}. The authors reported an overall reduction of 33–44%, with DHA among the enzymes most sensitive to Quadris®. In the table, the control and all three Quadris® treatments were assigned different significance groups from the control for DHA ($p < .05$). Because this study used a commercial formulation, its estimates refer to the tested Quadris® treatment, not necessarily to the active ingredient alone.

A complementary pattern was reported by Baćmaga et al. (2024:9-14), who studied Amistar 250 SC in sandy-clay soil at a field dose of 0.110 mg kg^{-1} and a polluting dose of 32.92 mg kg^{-1}. The experiment used three sampling dates: 30, 60, and 90 days. At the field dose, DHA was 28.359, 28.139, and 19.719 µmol TPF kg^{-1} dry soil h^{-1}, compared with control values of 28.050, 27.454, and 19.159 on those dates. At the polluting dose, DHA was 27.034, 26.424, and 19.073, corresponding to reported decreases of approximately 3.62%, 3.75%, and 0.45% relative to the controls. The authors found that the field dose increased DHA at all three sampling times, while the polluting dose decreased it. They also reported that incubation time accounted for most of the variation in enzyme activity (η^2 = 87.86–99.76% across the enzymes), whereas dose and the dose-by-time interaction had comparatively little influence. This is a much smaller DHA response than the one reported in some other studies, and it should be described as such rather than generalized to all concentrations or soils.

Time after application changes the interpretation of these findings. Bending et al. (2007) and Howell et al. (2014:197-198) observed early decreases followed by movement toward, or above, control levels. Wang et al. (2018) found that the high-concentration response remained evident at day 28. Boteva et al. (2022:382-392) reported that DHA was most sensitive early in the time profile, with the temporal pattern continuing across their 120-day experiment. Baćmaga et al. (2024:9-14), in contrast, found substantial differences

between sampling dates but comparatively small treatment-related DHA changes. A result at one date therefore cannot characterize the full response. Nor does a later return of DHA to the control establish that microbial community composition or other soil functions have also recovered.

Soil amendments, field conditions, and biological interventions

Soil amendments and field conditions can alter measured DHA independently of the fungicide effect. In the biochar study, biochar increased DHA, and azoxystrobin still reduced activity relative to matched biochar-only controls (Sopeña & Bending, 2013:1527-1529). The field-versus-laboratory vineyard study by Herrero-Hernández et al. (2015:85) likewise found higher DHA in spent-mushroom-substrate (SMS)-amended than unamended soil. The authors attributed this difference to the organic matter, available carbon, and microbial populations associated with the amendment. Under field conditions, mean DHA was higher in azoxystrobin-treated soils than in untreated soils across the two tested doses (LSD = 27.73, $p = .0001$); the increase was smaller in SMS-amended than in unamended soil. The authors also reported significant effects of sampling time, soil, and their interactions on DHA ($p < 0.0001$). These results are specific to the vineyard soil and SMS amendment, and do not establish that azoxystrobin stimulates DHA generally.

Manna et al. (2013: 2956–2957) found no significant effect of azoxystrobin on DHA in rice soil under outdoor ambient CO_2, ambient CO_2 in open-top chambers, or elevated CO_2 in open-top chambers. The study measured DHA from day 0 to day 50 and reported no effect of elevated CO_2 on DHA; azoxystrobin likewise had no effect on DHA, fluorescein diacetate hydrolase, or acid phosphatase activities, although it slightly inhibited alkaline phosphatase activity up to day 40. Its table gives DHA in µg TPF released g^{-1} soil day^{-1}, but the authors' reported overall interpretation is that there was no significant DHA response. Accordingly,

individual differences in table entries should not be recast as significant treatment effects.

Adak et al. (2020:1357–1358) studied fungicides in rice rhizosphere soil under field conditions across two years. Azoxystrobin was applied at 125 g active ingredient ha^{-1}. Although fungicide-treated soils had lower DHA than controls at some sampling points, the authors state that azoxystrobin's effect on DHA was not significant. They describe the azoxystrobin treatment as hindering DHA, while the broader microbial response was short-term and had recovered by day 30. This field study is relevant to agricultural conditions but is not directly equivalent to a laboratory soil-spiking experiment: it involved a crop rhizosphere, field variability, and several fungicide treatments.

Baćmaga et al. (2017:6-7) examined bioaugmentation of soil contaminated with azoxystrobin at 22.50 mg kg^{-1}. The laboratory experiment utilized two soil types (loamy sand and sandy loam) and assessed enzyme activity after 90 days of incubation, with and without inoculation using specific bacterial and fungal consortia. In uninoculated soils treated solely with azoxystrobin at 22.50 mg kg^{-1}, dehydrogenase activity was reduced relative to uncontaminated controls. Following the application of a bacterial consortium (comprising *Bacillus* strains) to contaminated loamy sand, dehydrogenase activity was 44% higher than in the soil contaminated with azoxystrobin but left uninoculated; in sandy loam, the corresponding increase achieved by the bacterial consortium was 15%. These contrasts reflect bioaugmentation outcomes rather than standalone fungicide effects and demonstrate that microbial inoculation can modify dehydrogenase activity in contaminated soils.

The biobed study by Kumari et al. (2019:845-848) is also not a conventional agricultural-soil experiment. It used rice-straw/corn-cob and peat/compost biomixtures to study degradation of

azoxystrobin and imidacloprid. Conditioning the biomixtures for 10 days increased microbial biomass and DHA; pesticide application to the conditioned mixtures did not show a negative impact on microbial parameters. These results are useful for understanding pesticide treatment systems, but biobed substrates should not be combined with field-soil DHA outcomes.

Conclusion

Soil dehydrogenase activity is a useful indicator of microbial oxidative processes because it is associated primarily with viable microbial cells and responds to changes in soil conditions. Its value lies in providing evidence about one important aspect of soil biological functioning; it should not be interpreted on its own as a direct measure of total microbial biomass, respiration, or overall soil health.

The meaning of a DHA result depends on both the soil context and the assay protocol. Substrate type, incubation conditions, moisture, aeration, units, and sampling time can affect the measured activity and limit direct comparisons among studies. Reliable interpretation therefore requires clear reporting of methods and consideration of the soil's properties and experimental conditions. These requirements are particularly important when synthesizing results across studies.

For research on azoxystrobin, DHA can help assess changes in soil microbial oxidative activity, but it should be considered alongside other biological and soil measurements. Consistent methods and transparent reporting will make it easier to distinguish treatment-related responses from variation due to soil conditions or measurement procedures. Taken together, these principles support a cautious interpretation of DHA and its use as one component of a broader assessment of soil responses to fungicide exposure.

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CHAPTER 5

DNA BARCODING IN PLANT SYSTEMATICS: SPECIES IDENTIFICATION AND NEXT-GENERATION APPROACHES

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INTRODUCTION

DNA barcoding is a molecular identification approach that aims to determine the identity of an organism from short, standardised DNA regions. Its modern framework took shape when Hebert et al. (2003) demonstrated that the mitochondrial cytochrome c oxidase subunit I (COI) region could be used for species identification in animals. Rather than replacing morphological identification, the approach has made it easier to test taxonomic identity, particularly in specimens that are incomplete, sterile, juvenile, processed or lack diagnostic morphological characters. The underlying principle of DNA barcoding is simple: a short sequence obtained from an unknown (query) specimen is compared with reliable, taxonomically verified reference sequences, and the result is interpreted together with morphology and other biological evidence (Hebert et al., 2003; Ratnasingham & Hebert, 2007).

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Early studies expected an ideal barcode to show sufficient variation among species, to be relatively conserved among individuals of the same species, to be amplifiable with the same primers across broad taxonomic groups, to yield short, high-quality sequences, and to be recoverable from partially degraded material such as museum and herbarium specimens. These criteria remain valid today; however, next-generation sequencing, mini-barcodes, metabarcoding and whole-plastome approaches have led to the notions of an "ideal length" or a "single universal locus" being reconsidered according to the application. In other words, current barcoding is shifting from the search for a single gene fragment towards building a scalable molecular identification system with a resolution appropriate to the research question (Hollingsworth et al., 2011; Rani et al., 2026).

This chapter first briefly summarises DNA barcoding through the markers used in the major groups of organisms, and then examines in detail why finding a standard barcode is more difficult in plants. The historical development of plant barcodes; the strengths and weaknesses of *rbcL*, *matK*, ITS/ITS2 and supplementary loci; the barcode gap; the distinction between species identification and species delimitation; reference infrastructures such as BOLD and GenBank; herbarium material; the authentication of medicinal and economic plants; metabarcoding; environmental DNA; and plastome-based "super-barcoding" approaches are discussed in the light of the current literature. This work is not a systematic review but a narrative and critical synthesis that considers landmark publications together with recent reviews from 2024–2026.

CONCEPTUAL BASIS AND HISTORICAL DEVELOPMENT OF DNA BARCODING

Barcode Regions in Different Groups of Organisms

Although the underlying logic of DNA barcoding is similar across all groups of organisms, the genomic region used is not the same. In animals, COI became the classical standard barcode because of its high copy number, sufficient interspecific variation in most groups and relatively easy alignment. In fungi, owing to the amplification and discrimination problems of COI, the nuclear ribosomal internal transcribed spacer (ITS) region was adopted as the primary barcode (Schoch et al., 2012). In bacteria and archaea, 16S rRNA sequences are a widely used identification tool, although resolution may be limited among closely related species and genomic approaches may be required. Because protists are an extremely heterogeneous group, there is no single universal species-level barcode; the variable regions of 18S rDNA are used as a pre-barcode, while COI, *rbcL* and other group-specific markers are used complementarily in different lineages (Antil et al., 2023; Pawlowski et al., 2012; Rani et al., 2026).

Table 1. Common DNA Barcode Markers in Major Groups of Organisms

Group	**Common marker**	**Genome/region**	**Key note**
Animals	COI	Mitochondrion	High species discrimination in many animal groups; complementary loci are needed in some groups.
Fungi	ITS	Nuclear ribosomal	Primary barcode for broad fungal diversity; additional genes may be needed for some closely related species.
Bacteria and archaea	16S rRNA	Ribosomal	Wide range of use; genomic resolution may be needed for closely related species and species boundaries.
Land plants	*rbcL* + *matK*; ITS/ITS2 supplementary	Plastid + nuclear	A single locus is usually insufficient; hybridisation and recent speciation reduce resolution.

Group	Common marker	Genome/region	Key note
Algae / protists	18S, COI, *rbcL*, tufA, etc.	Varies by group	Evolutionary diversity requires group-specific barcodes and a multi-step approach.

Source: Compiled by the author from Schoch et al. (2012), Antil et al. (2023) and Rani et al. (2026).

Properties Expected of an Ideal Barcode Region

The usefulness of a DNA region as a barcode does not depend on high variability alone. A successful barcode should show high PCR and sequencing success across broad taxonomic samples; its sequences should be easy to orient and align; it should not be excessively variable within a species; and it should provide sufficient discrimination among closely related species. Standardisation, identification from minimal data and scalability to large numbers of samples have been listed among the core design principles of plant barcoding (Hollingsworth et al., 2009; Hollingsworth et al., 2011). For herbarium, processed-product and environmental samples, the production of short amplicons becomes particularly important; therefore, alongside classical barcodes of approximately 500–900 base pairs, mini-barcodes of 100–300 base pairs have emerged as a practical solution (Nazar et al., 2025; Taberlet et al., 2007).

When interspecific variation is clearly greater than intraspecific variation, an identification window known as the "barcode gap" may arise. However, it cannot be assumed that this gap will be present in all taxa or at all loci. In plants in particular, hybridisation, polyploidy, chloroplast capture and incomplete lineage sorting may cause interspecific and intraspecific genetic distances to overlap. The performance of a barcode locus should therefore be assessed not only by mean distance values but also in relation to sampling density, the accuracy of reference sequences,

monophyly, identification success and biological context (Fazekas et al., 2009; Ho et al., 2026).

THE DNA BARCODING WORKFLOW

Sampling, Vouchers and Taxonomic Verification

DNA barcoding begins not with DNA extraction in the laboratory but with correct sampling and correct taxonomic identification. A reference sequence is only as reliable as the identity of the specimen to which it is linked. Wherever possible, each barcode sequence should therefore be associated with a collection number, locality, date, collector information, photographs and a herbarium voucher. In plant reference libraries in particular, herbarium specimens verified by experts are critical because they provide physical evidence that can be re-examined later. In national-scale barcode libraries, systematic sampling of herbarium collections improves both taxonomic quality and geographic coverage (Kartzinel et al., 2025; Whitley et al., 2024).

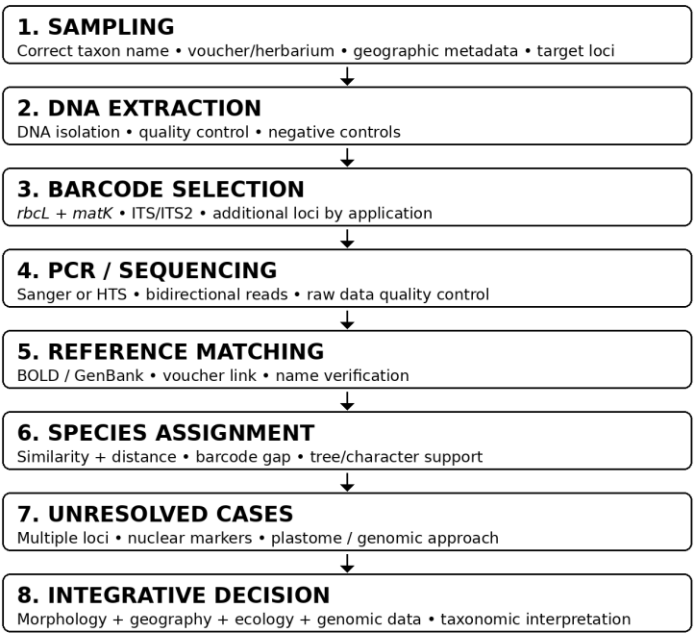
DNA Extraction, PCR and Sequencing

Although fresh leaf tissue is suitable for obtaining high-molecular-weight DNA in most plant species, secondary metabolites, polysaccharides and phenolic compounds can inhibit DNA extraction and PCR. Tissue rapidly dried in silica gel is a common option in fieldwork. Because DNA in herbarium specimens and commercial products is more fragmented, extraction conditions, amplicon length and contamination control are especially important. In classical barcoding projects, the PCR product is usually read by Sanger sequencing, whereas high-throughput sequencing platforms are preferred for mixed samples, studies involving hundreds of taxa, or environmental DNA (Kress, 2017; Whitley et al., 2024).

Raw Sequence Quality and Reference Matching

After sequencing, chromatogram quality should be checked, low-quality ends trimmed, bidirectional reads assembled and possible contamination screened. In protein-coding loci, unexpected stop codons or frameshifts may indicate off-target amplification or a pseudogene. In reference matching, it is not sufficient to look only at the highest BLAST percentage; the sampling level of the closest species, the discriminatory power of the region used, the overlap length between query and reference sequences, the voucher link and the current taxon name should all be considered together. BOLD is a barcode data system developed to bring molecular data together with specimen, morphological and distribution information, whereas GenBank is a broader-purpose nucleotide archive (Ratnasingham & Hebert, 2007).

Figure 1. Workflow of DNA Barcoding From Sampling to Integrative Taxonomic Decision



Source: Prepared by the author.

WHY IS DNA BARCODING MORE DIFFICULT IN PLANTS?

Low Substitution Rate in the Plant Mitochondrial Genome

The high success of COI in animals raised the expectation that the same locus could be used universally in plants; however, the lower nucleotide substitution rate of mitochondrial genes in land plants compared with many animal groups prevented COI from discriminating most plant species. Plant barcode research therefore turned to the plastid genome and nuclear ribosomal regions (Chase et al., 2005; Kress et al., 2005). In plants there is a strong trade-off between a genomic region being easily amplified with universal primers and evolving rapidly enough to separate closely related species.

Hybridisation and Chloroplast Capture

Interspecific hybridisation is more common in plants than in many animal groups, and because the plastid genome is inherited from a single parent in most angiosperms, interspecific gene flow can carry plastid lineages independently of species boundaries. This process can produce patterns known as “chloroplast capture”. As a result, two morphologically well-separated species may share the same plastid barcode, or geographically very different plastid lineages may occur within a single species. Fazekas et al. (2009) reported lower species monophyly and a smaller barcode gap in plant barcodes than in animals and suggested that hybridisation may be one of the main causes of this difference. In such cases, nuclear loci and morphological/ecological data are essential complements to plastid barcodes (Ho et al., 2026; Hollingsworth et al., 2016).

Polyploidy, Apomixis and Rapid Speciation

Polyploidy, allopolyploidy, apomixis and shifts in breeding system allow new taxa to arise in plants over relatively short evolutionary timescales. The fact that newly formed species are morphologically and ecologically differentiated does not mean that sufficient nucleotide divergence has accumulated in slowly evolving plastid loci such as *rbcL* or *matK*. In allopolyploids, the coexistence of nuclear copies derived from different progenitors can also complicate the interpretation of multicopy regions such as ITS. In closely related plant species, “barcode failure” may therefore be not merely a technical shortcoming but a genuine biological reflection of speciation history (Fazekas et al., 2009; Hollingsworth et al., 2011; Kumar et al., 2026).

Incomplete Lineage Sorting and Gene Tree–Species Tree Discordance

In recently diverged lineages, ancestral polymorphisms may persist in the new species for some time. Incomplete lineage sorting

causes the gene tree of a single locus to appear different from the true species tree. The sharing of a barcode region between two species therefore does not always indicate misidentification or ongoing hybridisation. Because standard barcodes provide a limited number of linked characters, resolution is reduced especially in rapid radiations. Although current genomic approaches aim to generate more characters from whole plastomes or many independent nuclear loci, a large number of characters derived from a single line of inheritance does not automatically resolve biological conflict; independent genomic compartments may need to be evaluated together (Hollingsworth et al., 2016; Shah et al., 2026).

CORE AND SUPPLEMENTARY BARCODE REGIONS IN LAND PLANTS

rbcL

rbcL is a plastid gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and has long been used in plant phylogenetics. Its high primer universality and sequence quality make it advantageous for large-scale reference libraries. By contrast, its variability among closely related species is often low. *rbcL* has therefore been regarded as an important component of the core barcode because of its reliable recovery and higher-level taxonomic placement rather than high species resolution on its own (CBOL Plant Working Group, 2009; Hollingsworth et al., 2011).

matK

matK is a rapidly evolving region located within the plastid *trnK* intron that encodes a maturase. It can provide higher species discrimination than *rbcL* in most groups; however, the unequal performance of universal primers across land plants was long a significant technical problem. Taking into account the balance between recoverability, sequence quality and discriminatory power,

the CBOL Plant Working Group (2009) proposed the *rbcL* + *matK* pair as the core barcode for land plants. Lahaye et al. (2008) reported that *matK* alone could achieve high identification success in the floras of biodiversity hotspots. This decision does not imply that every species will be unambiguously separated; rather, it provides a common starting framework for international standardisation.

ITS and ITS2

The ITS region of nuclear ribosomal DNA is a powerful complement, particularly for discriminating closely related species, because it can evolve faster than plastid genes and carries a biparentally inherited nuclear signal. Using a large dataset, the China Plant BOL Group (2011) supported the incorporation of ITS into the core barcode for seed plants. A large-scale study of medicinal plants reported that ITS2 can achieve high identification success (Chen et al., 2010). Nevertheless, ITS is multicopy; paralogues, incomplete concerted evolution, fungal contamination and double peaks during sequencing can cause problems of interpretation. Reviewing 197 angiosperm barcoding studies in 2025, Aygören Uluer emphasised that ITS2 performs well in amplification and genus-level identification, but that species-level success is not universal and that none of the regions examined can be considered a universal barcode for all angiosperms on its own (Aygören Uluer, 2025).

***trnH-psbA*, *trnL* and Other Supplementary Regions**

Because of its high variability, the plastid *trnH-psbA* intergenic spacer was, together with *rbcL*, among the early proposals for a plant barcode (Kress & Erickson, 2007). However, length variation, inversions, homopolymer regions and alignment difficulties can cause problems in broad phylogenetic samples. The short P6 loop of the *trnL* intron, despite its lower species discrimination, has become an important mini-barcode for metabarcoding of faeces, soil, ancient material and processed

samples because it yields very short amplicons (Deiner et al., 2017; Taberlet et al., 2007). In certain taxonomic groups, *ycf1*, plastid spacers other than *trnH-psbA*, or group-specific nuclear loci may also complement the standard core barcodes (Dong et al., 2015).

Table 2. Comparison of Common Barcoding Approaches in Plants

Marker	**Recovery**	**Species resolution**	**Main advantage**	**Main limitation**
rbcL	High	Low–moderate	Universality and high sequence quality	Insufficient variation among close relatives
matK	High in angiosperms; variable in other groups	Moderate–high	More variable than *rbcL*; core barcode	Low primer universality in some groups
ITS/ITS2	Moderate–high (high for ITS2)	Moderate–high	Nuclear, rapidly evolving signal	Paralogues, intragenomic variation, contamination
trnH-psbA	High	Moderate–high	Variable spacer; easy amplification	Indels, inversions and alignment problems
trnL P6	Very high	Low–moderate	Very short; suitable for degraded DNA and eDNA	Limited species-level discrimination
Plastome / super-barcode	Depends on genomic data	High	Many characters; design of group-specific mini-barcodes	Cost, data analysis; biological limits of a single plastid lineage

Source: Compiled by the author from CBOL Plant Working Group (2009), Hollingsworth et al. (2011) and Li et al. (2015).

BARCODE GAP, SPECIES IDENTIFICATION AND SPECIES DELIMITATION

Interpreting the Barcode Gap

One of the most widely used concepts in DNA barcoding is the barcode gap, which rests on the expectation that intraspecific genetic distances are lower than interspecific distances. A clear gap facilitates identification; however, the threshold value is not

expected to be constant across all taxa. Insufficient sampling, geographic structure, introgression and misidentified reference specimens can artificially widen or narrow the gap. A percentage similarity or a single genetic distance threshold should therefore not be used as an automatic species decision, especially in plants (Fazekas et al., 2009; Hollingsworth et al., 2011; Meyer & Paulay, 2005).

Species Identification and Species Delimitation Are Not the Same Process

Species identification aims to assign an unknown specimen to a previously described taxon, whereas species delimitation seeks to determine the boundaries of evolutionarily independent lineages. High similarity between a query sequence and a reference species may provide evidence that the specimen is consistent with that species; however, it does not by itself demonstrate the existence of a new species or that two closely related taxa are independent species. Early debates over whether DNA barcoding should replace classical taxonomy have largely been superseded by the integrative taxonomy approach, which combines morphology, geography, ecology and multiple sources of genetic data (Dayrat, 2005; DeSalle et al., 2005; Will et al., 2005).

Neighbour-joining trees or barcode clusters are useful visualisations for identification and for revealing potential cryptic diversity; however, they are not synonymous with detailed phylogenetic analysis. In particular, high similarity in a single-locus barcode tree does not fully resolve the shared evolutionary history of species. When species delimitation is required, hypotheses should be tested with independent nuclear loci, genomic SNP data, coalescent-based methods and phenotypic/ecological evidence (DeSalle et al., 2005; Will et al., 2005).

REFERENCE LIBRARIES: BOLD, GENBANK AND THE VOUCHER PROBLEM

The success of a barcoding system depends not only on a good choice of marker but also on the accuracy of the reference library against which the query is compared. BOLD is a dedicated system that aims to link barcode sequences with vouchers, images, geographic coordinates and collection data (Ratnasingham & Hebert, 2007). GenBank, in contrast, is a much broader sequence archive that also contains genomic data beyond barcodes. Both systems are indispensable; however, users should check whether taxonomic names are up to date, which physical specimen a sequence is based on, and whether the identification has been verified by an expert.

Evaluating more than 373,584 plant barcodes in global public databases in 2025, Kartzinel et al. reported that these records cover only about a quarter of the approximately 435,000 extant land plant species and that at least 17% of plant families have no reference barcode at all. This result clearly shows that even a technically flawless metabarcoding protocol cannot produce species-level results when suitable reference sequences are lacking. Moreover, the fact that most specimens originate from the Global North leads to geographic and taxonomic gaps in tropical biodiversity hotspots (Kartzinel et al., 2025).

The Role of Herbaria in Reference Barcode Libraries

Herbaria are unique for reference barcode libraries because they hold millions of specimens whose names have been determined by experts and which can be re-examined. They make it possible to generate barcodes without new field collections, especially for rare, endemic or currently inaccessible species. However, age, drying method, chemical treatment and storage conditions can increase DNA fragmentation. Short amplicons, multiple short regions, target

enrichment and high-throughput sequencing are therefore becoming increasingly important in herbarium barcoding (Whitley et al., 2024).

APPLICATIONS OF DNA BARCODING

Biodiversity Inventories, Conservation and Ecological Monitoring

DNA barcoding provides rapid preliminary identification in large biodiversity inventories where morphological identification is time-consuming or expertise is scarce. Species can be assigned to material lacking diagnostic morphological characters, such as seedlings, seeds, roots, wood fragments, pollen or leaf fragments. Important applications include the identification of endangered and trade-regulated species in products or parts, the early detection of invasive species and the resolution of ecological interaction networks (Kress, 2017; Rani et al., 2026). Nevertheless, in studies that inform conservation decisions, the accuracy of the reference library is especially critical, because false-positive or false-negative species assignments may have legal and management consequences.

Agricultural, Food and Forensic Applications

Accurate identification of agricultural pests and pathogens, import–export controls and the authentication of plant-derived food products are applied areas of barcoding. In materials whose morphological characters have been lost, such as ground spices, herbal teas or processed foods, DNA-based methods are powerful for label verification and the detection of adulteration. A comprehensive 2024 review emphasised that DNA barcoding is widely used to authenticate plant-derived food, feed and medicinal products, and that ddPCR, isothermal amplification and metabarcoding have expanded the toolkit in this field (Lanubile et al., 2024).

Authentication of Medicinal Plants

In medicinal plants, the use of the wrong species, synonyms, local names that refer to more than one taxon, morphologically similar adulterants and the loss of diagnostic characters in processed material are major quality problems. The success of ITS2 in identifying medicinal plants was demonstrated early on a large sample, and mini-barcode, metabarcoding and plastome-based approaches have since advanced the field (Chen et al., 2010; Chen et al., 2023). However, DNA does not measure the amount of active chemical constituents or pharmacological quality. In modern quality assurance, an orthogonal approach in which DNA barcoding and morphological, microscopic and chemical profiling methods corroborate one another is therefore considered more robust (Nazar et al., 2025).

MINI-BARCODING, METABARCODING AND ENVIRONMENTAL DNA

Mini-Barcodes

Amplification of full-length standard barcodes may fail in old herbarium specimens, boiled crude drugs, capsule/tablet contents and highly processed products. Mini-barcodes increase the likelihood of obtaining results from fragmented DNA by using short, informative subregions that discriminate the target species. The *trnL* P6 loop is a classic example in plant eDNA and diet studies because of its very short inter-primer length; however, a short sequence also reduces the number of characters that discriminate at species level (Taberlet et al., 2007). In mini-barcode design, a balance must therefore be struck between amplification success and discriminatory power.

DNA Metabarcoding

Metabarcoding aims to identify many organisms present in a single sample by simultaneously amplifying their barcode regions with universal or broad-range primers and sequencing them by high-throughput sequencing. Soil, water, pollen, honey, faeces, stomach contents, plant mixtures and commercial preparations are typical examples of this approach. Unlike classical barcoding, metabarcoding usually works on a mixed DNA pool rather than a single clean DNA template. Primer bias, copy number in different species, amplicon length, sequencing depth and bioinformatic filters can therefore affect the interpretation of the relative abundances of species (Deiner et al., 2017).

Environmental DNA (eDNA)

Environmental DNA makes it possible to investigate the presence of an organism from the DNA it releases into water, soil, air or other environmental media, without the need to capture the organism directly. In plants, eDNA offers opportunities for community-level monitoring through pollen, root material, shed cells and particles. Although eDNA and metabarcoding scale up biodiversity monitoring, contamination, transported DNA, the persistence of DNA in the environment and gaps in reference libraries can complicate the ecological interpretation of a “presence” signal. Laboratory and field negative controls, replicates and explicit bioinformatic thresholds are therefore essential (Deiner et al., 2017; Rani et al., 2026).

FROM CLASSICAL BARCODES TO PLASTOME AND GENOMIC BARCODING

Super-Barcoding and Plastome Sequencing

The limited performance of the standard *rbcL* + *matK* system among closely related species has brought the use of the whole chloroplast genome as a “super-barcode” onto the agenda. Because

plastomes contain far more variable characters than standard barcodes, they can markedly improve the discrimination of closely related species when appropriate reference specimens are available. Li et al. (2015) proposed that single-locus and plastome approaches be used not as alternatives but as complementary tools depending on the taxon and the scale of application. In recent years, falling sequencing costs have made the routine production of plastomes through genome skimming easier, and it has also become possible to select shorter, lineage-specific, highly variable mini-barcodes from plastomes (Hollingsworth et al., 2016; Shah et al., 2026).

The availability of more characters at the plastome scale does not mean that all species-boundary problems will be solved. If chloroplast capture or ongoing hybridisation is present, the whole plastome represents the same line of inheritance and may reproduce an incorrect species relationship with high support. In closely related, reticulately evolving or polyploid groups, nuclear single- or low-copy genes, target enrichment or genome-wide SNPs should therefore be evaluated together with plastome data (Ho et al., 2026; Hollingsworth et al., 2016).

Long-Read Sequencing and Machine Learning

Long-read platforms such as Nanopore and PacBio facilitate the rapid recovery of longer amplicons, complete ribosomal regions and, in some cases, organelle genomes. These technologies have considerable potential for field-based sequencing and real-time identification (Srivathsan et al., 2021); however, raw-read error profiles, reference library quality and analytical standardisation must be managed carefully according to the application. Machine learning approaches can learn classification patterns from high-dimensional sequence data; however, model performance depends on the taxonomic accuracy and representativeness of the training dataset (Shah et al., 2026). Recent reviews from 2026 regard the future of

plant barcoding not as a single locus but as a “genomic toolkit” in which classical markers are used together with plastomes, multiple loci, metabarcoding and computational classification (Ho et al., 2026; Kumar et al., 2026; Shah et al., 2026).

Table 3. Major Problems in Plant DNA Barcoding and Suitable Solutions

Problem	**Possible consequence**	**Recommended approach**
Recent speciation / incomplete lineage sorting	The same barcode haplotype shared by more than one species	Denser sampling, independent nuclear loci, genomic species delimitation
Hybridisation / chloroplast capture	Plastid tree inconsistent with species boundaries	Nuclear markers + morphology + geographic/ecological evidence
Polyploidy and paralogues	Multicopy signal in ITS and nuclear sequences	Copy awareness, target enrichment, genomic data
Degraded DNA	PCR failure of long barcodes	Mini-barcodes, short amplicons, HTS
Incomplete reference library	Species-level assignment not possible	Voucher-based national/regional reference libraries
Mixed sample	Overlapping signal in the Sanger sequence	Metabarcoding with appropriate negative/positive controls
Incorrect reference name	Wrong species assignment with high similarity	Voucher re-examination, taxon name updating, multiple databases

Source: Prepared by the author.

WHAT DNA BARCODING IS NOT

DNA barcoding is not an “automated taxonomy” that replaces the existing body of taxonomic knowledge. Without correctly named reference specimens, the barcode system itself cannot provide reliable identification. Moreover, a single barcode tree is not synonymous with a phylogeny; resolving historical

relationships among species requires model selection, multiple gene regions or genomic data, and appropriate outgroup sampling. Likewise, high sequence similarity is not by itself sufficient evidence for describing a new species or for a species delimitation decision (DeSalle et al., 2005; Will et al., 2005).

The most powerful use of DNA barcoding is the rapid identification of unknown specimens against references verified by expert morphological identification, the detection of discordant specimens, and the flagging of lineages that require more detailed taxonomic study. Deep genetic divergence in a barcode dataset may raise a new species hypothesis; however, any nomenclatural decision should be supported by type material, morphology, distribution, ecology and, where possible, independent genetic evidence. This approach makes DNA data not a rival to classical systematics but a powerful component of integrative taxonomy (Dayrat, 2005; Kress, 2017).

CURRENT STATUS AND FUTURE PERSPECTIVES

Since 2003, DNA barcoding has evolved from the idea of COI-based standard identification into a molecular identification ecosystem operating at very different scales as of 2026. The standardisation provided by a single powerful mitochondrial locus in animals has never been achieved to the same extent in plants; this limitation reflects the reticulate and multi-genomic nature of plant evolution rather than a weakness of plant barcoding. The current approach is not to abandon standard core loci such as *rbcL* and *matK* but to complement them stepwise, according to the application, with ITS/ITS2, short mini-barcodes, whole plastomes and independent nuclear markers (Ho et al., 2026; Kumar et al., 2026).

The most important need in the coming years is not simply to produce more sequences but to produce the right sequences from the right specimens. The large geographic and taxonomic reference gaps

demonstrated by Kartzinel et al. (2025) increase the importance of expert-verified libraries based on herbarium and living collections if new sequencing technologies are to be used efficiently. Recording barcodes in national flora projects according to an “extended specimen” concept, together with the current taxon name, voucher, photographs, distribution and, where possible, genomic data, will provide a shared infrastructure for basic systematic research, eDNA monitoring and forensic/economic applications.

For plant systematics, the future lies in a decision-tree approach rather than the search for a single “best barcode”. Standard barcodes may be economical and sufficient for routine authentication; ITS and additional plastid/nuclear loci may come into play for closely related species; many independent nuclear regions are needed when hybridisation or polyploidy is suspected; mini-barcodes and metabarcoding come to the fore for processed or mixed material; and plastome and genomic data can be used for groups that standard barcodes cannot resolve. This stepwise strategy balances cost and resolution while also preventing biological over-interpretation of the results.

CONCLUSIONS AND RECOMMENDATIONS

DNA barcoding is a powerful molecular tool, complementary to classical taxonomy, that evaluates organismal identity from short, standardised DNA sequences. The differences in the loci used across groups of organisms stem from differences in genomic evolutionary rates and modes of inheritance. While COI is common in animals, ITS in fungi, 16S rRNA in bacteria and archaea, and group-specific combinations in protists, in land plants the *rbcL* + *matK* core system is supported by ITS/ITS2 and other supplementary markers. In plants, low mitochondrial substitution rates, hybridisation, chloroplast capture, polyploidy and incomplete lineage sorting have hindered the development of a single universal species barcode.

The most reliable approach in plant barcoding is therefore a stepwise workflow that begins with a correctly identified voucher specimen, proceeds through appropriate locus selection and sequence quality control, uses more than one reference source, and integrates the results with morphological/ecological evidence. While standard loci retain their importance in routine identification, mini-barcodes for herbarium and processed material, metabarcoding for mixed samples, and plastome and independent nuclear genomic approaches for closely related species are increasing resolution. However advanced the technology becomes, the value of a barcode sequence is limited by the correctly named specimen it represents and by a reliable reference library. The future of DNA barcoding in plant systematics is therefore taking shape within an integrative framework in which molecular speed and classical taxonomic accuracy advance together.

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