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Hazard identification and the harmful effects of organophosphates and carbamates in bioethical models: a review

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Pesticides are an emerging category of contaminants employed in agriculture or for domestic use. Among them, anti-cholinesterase insecticides are the most extensively applied. These are neurotoxic compounds released into the environment in an estimated millions of tons per year, posing serious risks to both environmental and human health. This review examines the current literature on hazard identification and the assessment of two major classes of neurotoxic compounds—organophosphates (OPs) and carbamates (CBs)—in relation to human and environmental health. We report the harmful effects of OPs and CBs on humans and present bioethical models as alternatives to vertebrates in toxicological testing. We describe the most utilized bioassays, employing a range of experimental models (cells, monolayer cultures, spheroids, and whole organisms) exposed to different doses of OPs and CBs, to identify and quantify the most sensitive biological responses (endpoints and biomarkers). This novel approach aims to improve predictive capacity and strengthen the risk assessment of human and environmental health. In conclusion, *in vitro* or non-mammalian models are promising alternatives for assessing human health risks caused by OPs and CBs, in line with the "Replacement, Reduction, and Refinement" (3R) principle.

KEYWORDS
bioassays, bioethical models, biomarkers, carbamates, organophosphates

1 Introduction

Recently, a new class of substances has been identified as chemicals of high concern: so called "emerging contaminants" (ECs). They include synthetic or naturally occurring chemicals, as well as microorganisms, that are not routinely monitored in the environment but may enter it and pose potential risks to both ecological and/or human health (Rosenfeld and Feng, 2011). ECs include pharmaceuticals, personal care products, endocrine-disrupting compounds, nanomaterials, and industrial chemicals such as pesticides, which can reach the environment through multiple pathways (Li et al., 2024). Once released, ECs may accumulate along the food chain, threatening terrestrial and aquatic ecosystems and, ultimately, human health. Pesticides are a class of ECs widely applied worldwide. They are used repeatedly throughout the year in various forms (i.e. powder, aerial sprays, and through irrigation), resulting in their persistence in the environment for prolonged periods and at relatively high concentrations. It is currently estimated that more

than 3.5 million tons of pesticides are poured into the environment annually (Sharma et al., 2019) with noxious effects on environmental and human health. The action of pesticides is based on poisoning insects and small vertebrates, but this effect is also exerted on other non-target organisms, reached through different pathways (Figure 1). Pesticides employed in agricultural sites or for domestic use reach soil, surface water and groundwater, crops, and all organisms that feed, drink, and breathe in the treated area, thus finding their last target in human beings. Most pesticides are soluble in lipids, so they accumulate in edible organisms to become bioavailable for consumers and predator species.

Most pesticides enter the environment accidently or as byproducts of industrial, mining, or domestic activities (e.g., smoke, waste, and micro- and nanoparticles). Others are intentionally released due to their toxicity to pest control in agricultural and farming systems. These pesticides are used to protect crops from insects, fungi, weeds, and vertebrates that negatively impact global agricultural production. Because pesticides can follow multiple pathways and human exposure may occur through inhalation, dermal contact, and ingestion, much recent research has focused on hazard identification and effect assessment within the "Human Health Risk Assessment" (HHRA) framework. Based on their mode of action (MOA) as defined by IRAC (2020), pesticides are broadly grouped into two classes: endocrine disrupters and neurotoxic pesticides. Endocrine-disrupting pesticides are represented by DDT [para-dichloro-diphenyl-trichloroethane ($C_{14}H_9Cl_5$)], widely used from the mid-20th century for domestic and agricultural pest control. DDT disrupts endocrine function, leading to developmental, reproductive, neurological, and immune effects in humans and wildlife (Dalvie et al., 2004; McKinlay et al., 2008; Bienkowski, 2015). Due to its toxicity and environmental persistence, it was banned in the USA in 1972 and is classified as a "persistent organic pollutant." Its production and use were subsequently prohibited under the Stockholm Convention in 2001, signed by 170 countries (EPA, 2004). The class of "neurotoxic pesticides" refers to those compounds that mainly affect nervous functions. They are still used in agriculture, but some—organophosphates (OPs) and carbamates (CBs) —are under observation for possible risk to aquatic organisms, wild fauna, and human health.

This review reports the characterization of the source, the MOA related to the chemical and physical properties of OPs and CBs, and the risks posed to human and environmental health. The novelty of this review is to identify the hazards and the effects of OPs and CBs in bioethical models as alternatives to vertebrates in toxicological testing, in accordance with the 3R ("Replacement, Reduction, and Refinement") principle. The literature on the harmful effects of OPs and CBs *in vitro* in non-mammalian models is thus systematic reviewed to verify whether such models and the different measurable biomarkers can be evaluable alternatives to enhance the risk assessment of human and environmental health.

2 Neurotoxic pesticides

The neurotoxic pesticides OPs and CBs are employed for many purposes, including agricultural, garden, and even domestic pest control. OP compounds have been studied for a wide range of aims, from chemical weapon to pest control and medical compounds (anxiolytic, antispasmodic, regulators of eye pressure, etc.; Costa, 1987).

Both pesticides inhibit cholinesterase (ChE) (acetylcholinesterase, AChE, E.C. 3.1.1.7) and pseudo-cholinesterase (e.g., propionylcholinesterase PrChE E.C. 3.1.1.8.; butyrylcholinesterase BuChE, E.C. 3.1.1.8) activities, altering the status of the cholinergic neurotransmitter and neuromuscular system (Falugi et al., 2011). Specifically, ChE inhibition causes an overflow of acetylcholine (ACh) at receptor sites that, in turn, affect intracellular responses. AChE and pseudo-cholinesterase activity are not the only target of CB and OP toxicity (Casida and Durkin, 2013); secondary non-AChE targets, such as muscarinic ones, can be directly affected due to ACh receptor inhibition (Aluigi et al., 2005; Lotti and Moretto, 2005; Aluigi et al., 2010a, b). Differences between CBs and OPs occur in their interaction with AChE. Both neurotoxic compounds bind to the serine residue in the enzyme's catalytic "gorge", but OPs phosphorylate the hydroxyl residue and remain tightly bound, causing long-lasting inhibition (Figure 2). CBs, instead, reversibly carbamylate the serine hydroxyl residue, temporarily displacing ACh and allowing rapid enzyme recovery (Alvares, 1992). Environmentally, OPs can leave persistent residuals, while CBs break down into small inorganic molecules (e.g. carbon dioxide). These compounds also differ in their molecular mass and persistence: OPs are larger (approximately 300 Da) than CBs (200 Da) and exhibit a longer half-life on crops, contributing to prolonged AChE inhibition compared to CBs.

2.1 Persistence and half-life

The persistence of OPs and CBs has been evaluated through laboratory experiments and theoretical models to estimate their environmental and occupational presence. Knowledge of their half-life, soil adsorption, and water solubility helps regulative bodies prevent environmental and human health risks (Lewis et al., 2016). Based on their half-life, these pesticides are classified as follows.

- Non-persistent, degrade by half in <30 days.
- Moderately persistent, degrade by half in 30–100 days.
- Persistent, degrade by half in >100 days.

The persistence of OPs and CBs in the environment is generally considered short, but these neurotoxic compounds can persist in sediments and water bodies, causing mortality among fish, mollusks, and aquatic birds (Giger, 2007). OPs with short half-lives can persist up to 1 year under low pH and temperature (Ragnarsdottir, 2000), probably due to adsorption to soil particles that prevents microbial degradation. In living organisms, OPs may affect blood AChE for several months (Midtling et al., 1985). The persistence of CBs, like that of OPs, depends on soil and environmental conditions, such as pH. Thus, CBs with short half-lives (7 days) may persist in soil up to 12 days at pH values above 12, but as long as 560 days at pH 6.0 (Mink et al., 1989). The persistence of CBs can also increase or decrease in the aquatic environment. Diazinon and chlorpyrifos, for instance, remain in freshwater and seawater much longer than other CBs (e.g., malathion and carbaryl). In freshwater, microbial activity contributes significantly to the degradation of diazinon and

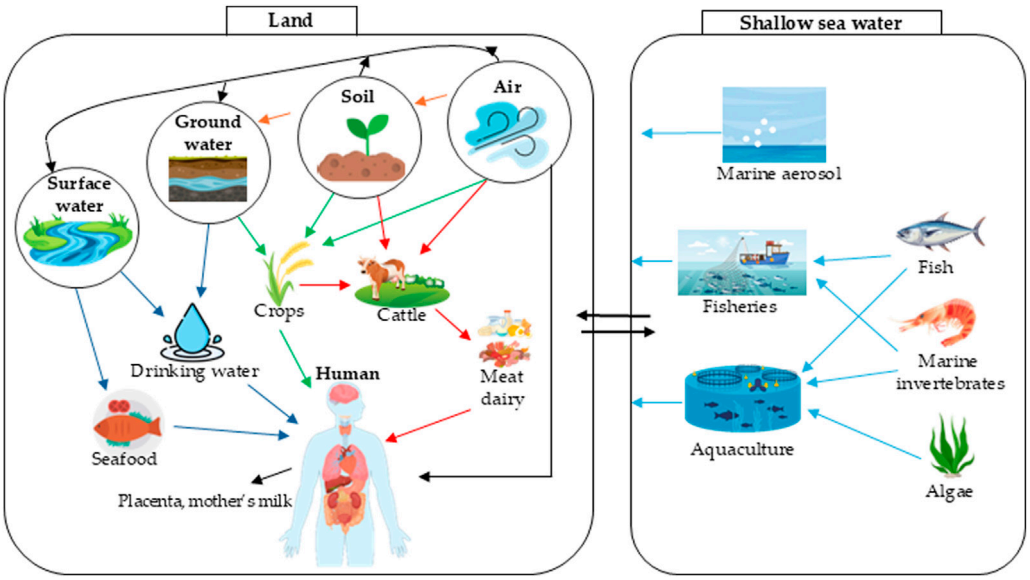

FIGURE 1
Distribution routes of pesticides from agricultural sites to humans. Through the air, pesticides used for domestic or agriculture purposes reach the soil, surface water, and groundwater, directly affecting non-target organisms (e.g., seafood, crops, and cattle) and indirectly humans. Since most pesticides are hydrophilic, they also accumulate in edible aquatic species (e.g. fish and marine invertebrates such as mussels and crustaceans) and are bioavailable for consumers and predators.

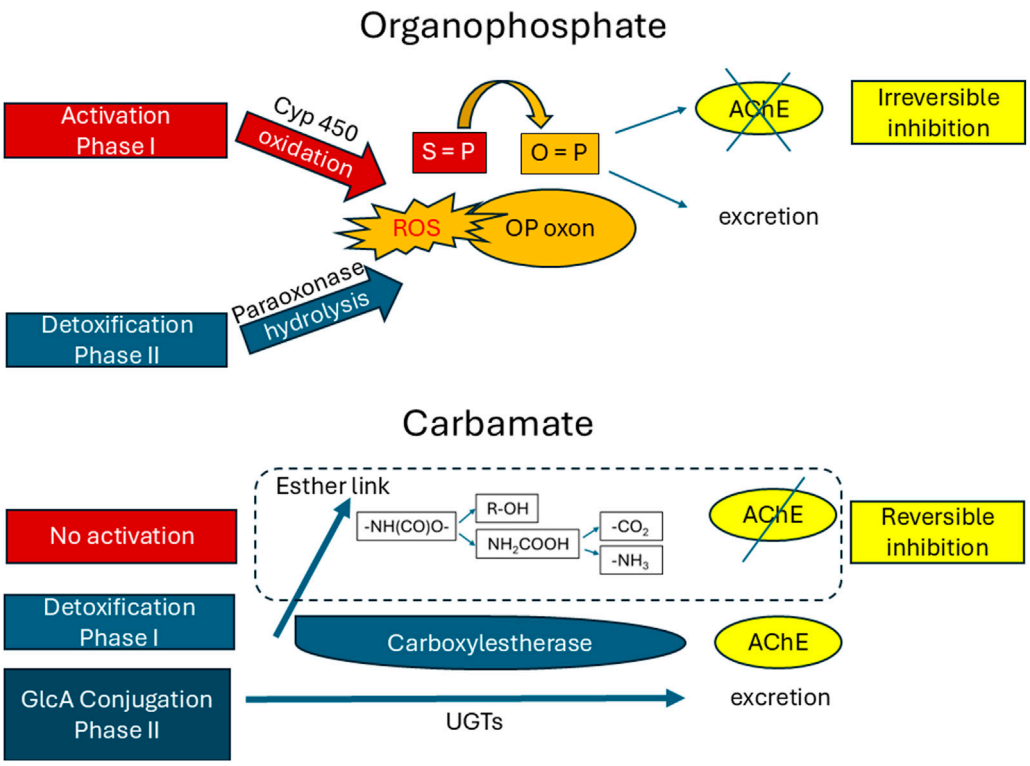

FIGURE 2
Scheme of the metabolism of organophosphate and carbamate pesticides. Cyp 450, cytochrome P450; UGTs, UDP-glucuronosyltransferases.

chlorpyrifos; however, this microbial degradation is inhibited in seawater, resulting in greater persistence (Bondarenko et al., 2004). Both CBs and OPs are lipophilic, enabling them to cross cell membranes and biological barriers (brain–blood and placental) and accumulate in body fat, plant oils, and milk—including human milk (Rakonczay and Papp, 2001; Lee et al., 2014).

2.2 Metabolism

Most OPs used as agricultural insecticides reach terrestrial and aquatic biota through inhalation, dermal contact, and ingestion (Nkinsa et al., 2020). Once inside the organism, they are metabolized by hepatic detoxification enzymes and converted into highly toxic oxon derivates (metabolites). During Phase I metabolism, cytochrome P450 oxidases replace sulfur atoms with oxygen, generating highly reactive intermediates (ROS). Generally, ROS are immediately neutralized by Phase II enzymes; however, oxon metabolites bind irreversibly to the active site of cholinesterases, fully inhibiting AChE activity (Tang et al., 2006). The oxon compounds are detoxified and excreted via urine (Camacho-Pérez et al., 2022). Conversely, CBs are metabolized in the liver through hydrolysis, hydroxylation, dealkylation, or S-oxidation (Rakonczay and Papp, 2001). Importantly, CBs do not undergo the "aging" process seen in the OP phosphorylation of AChE, and the CB-cholinesterase bond spontaneously hydrolyzes within hours, allowing the rapid restoration of enzyme activity (Tang et al., 2006).

2.3 Hazard identification

This step consists in:

- source characterization/identification;
- pollutant characterization/identification;
- hazard evaluation (usually by comparing field data with law limit values).

Pesticide sources are relatively simple to identify as they are voluntarily released into the environment. They can be classified as spatially extended, pseudo-continuous, or discrete, depending on the duration and frequency of application, and may also include production sites where industrial accidents or operational errors occur.

OPs and CBs are considered primary pollutants, although secondary contaminants can be formed through environmental transformation. Their characterization also considers the adverse effects they may cause, such as toxicity or carcinogenicity. Hazard evaluation shows that neurotoxic pesticides are strongly suspected of causing acute toxicity to human health at high doses, such as in accidents or occupational exposure. Chronic, low-dose effects—through aerosol or residuals in crops and vegetables—are more uncertain but raise public concern, especially when combined with other pollutants (e.g., heavy metals, and hydrocarbons; Pathak et al., 2022). Reference doses, like NOEC (no-effect concentration for humans) are often based on animal studies and may underestimate risks, particularly in sensitive stages such as embryonic development. Thus, research on neurotoxic pesticide effects and hazard assessment remains active and constantly evolving. The toxicity of neurotoxic compounds is well established, and risk (R) is usually calculated as the combination of toxicity (T) and exposure (E):

$$R = T \times E,$$

where R is the likelihood of harm occurring, T refers to the hazard as the potential to cause harm, while E is the contact level, according to the U.S. Environmental Protection Agency (1989) and the European Chemical Agency (ECHA). Toxicity can be determined and quantified through specific indices (such as LC/LD_{50} or EC_{50}) which indicate the concentration or dose of a compound that causes a measurable response in 50% of a test population. Exposure can be characterized based on the administered dose and the frequency with which neurotoxic compounds are used.

Different risk values may be found within the same target or non-target organisms exposed to a single neurotoxic compound, depending on whether acute, sub-chronic, and chronic toxicity is assessed and on the biological level of organization (i.e. molecule, cell, tissue, and organism) at which effects are measured. Moreover, risk values can vary depending on whether toxicity is mediated through interaction with the primary OP and CB target (AChE) or through secondary targets (pseudo-cholinesterase, nicotinic, and muscarinic receptors). Thus, it is essential to establish multiple risk values derived from a broad range of measurable endpoints that can serve as toxicity biomarkers. This approach enables a more comprehensive assessment of the risk of a single pesticide on target and non-target organisms, as well as on the surrounding environment, supporting the development of more protective regulatory strategies.

According to the Insecticide Resistance Action Committee (IRAC) classification, the anti-AChE insecticides are as follows.

CBs: alanycarb, aldicarb, bendiocarb, benfuracarb, butocarboxim, butoxycarboxim, carbaryl, carbofuran, carbosulfan, ethiofencarb, fenobucarb, formetanate, furathiocarb, isoprocarb, methiocarb, methomyl, metolcarb, oxamyl, pirimicarb, propoxur, thiodicarb, thiofanox, triazamate, trimethacarb, XMC, and xylylcarb;

OPs: acephate, azamethiphos, azinphos-ethyl, azinphosmethyl, cadusafos, chlorethoxyfos, chlorfenvinphos, chlormephos, chlorpyrifos, chlorpyrifos-methyl, coumaphos, cyanophos, demeton-s-methyl, diazinon, dichlorvos/ddvp, dicrotophos, dimethoate, dimethylvinphos, disulfoton, EPN, ethion, ethoprophos, famphur, fenamiphos, fenitrothion, fenthion, fosthiazate, heptenophos, imicyafos, isofenphos, isopropyl o-(methoxyaminothio-phosphoryl) salicylate, isoxathion, malathion, mecarbam, methamidophos, methidathion, mevinphos, monocrotophos, naled, omethoate, oxydemeton-methyl, parathion, parathion-methyl, phenthoate, phorate, phosalone, phosmet, phosphamidon, phoxim, pirimiphos-methyl, profenofos, propetamphos, prothiofos, pyraclofos, pyridaphenthion, quinalphos, sulfotep, tebupirimfos, temephos, terbufos, tetrachlorvinphos, and thio.

The toxicity of the most used CBs and OPs toward biota is described in the next paragraphs.

2.4 Toxicity

The toxicity of OPs and CBs can be classified according to:

i. their ability to damage organisms (Table 1; Nesheim et al., 2005; Damalas and Koutroubas, 2016);
ii. the power of inhibition, in terms of IC50 value (meaning of ChE inhibition in the 50% exposed populations) of ChE activities.

This power may change according to different models; for instance, demonstrating that electric eel (*Electrophorus electricus)* AChE is very resistant to OP inhibition compared to rat brain or *Drosophila melanogaster* brain (i.e. IC_{50}: 11 ± 1 μM in *E. electricus* vs. 1.06 ± 0.47 μM in *D. melanogaster* vs. 1.21 ± 0.11 μM in rat for chlorpyrifos; 51.0 ± 3.6 in *E. electricus* vs. 9.68 ± 2.61 μM in *D. melanogaster* vs. 27.00 ± 0.06 μM in rat for malathion; Rakonczay et al., 2006).

According to these classifications, the main OPs and CBs can be identified in different toxicity classes (Tables 2, 3 and Supplementary Tables).

2.5 Effect assessment

The effects of exposure to any toxic substance depend on the dose, duration, exposure mode, personal traits, habits, and the presence of other chemicals. An important step of human health risk assessment is to evaluate the effects of pesticides in animals and humans and to define the values of parameters such as PNEC, NOAEL, LOAEL, EC_{50}, and LC_{50} to be used to compute risk. Toxicity is classified as follows.

TABLE 1 Types of acute toxicity measures and warnings based on neurotoxic pesticide's ability to damage organisms.

Category	Signal word	Oral (mg/kg)	Dermal (mg/kg)	Inhalation (mg/L)
I Highly toxic	Poison	0–50	0–200	0–0.2
II Moderately toxic	Warning	50–500	200–2,000	0.2–2
III Slightly toxic	Caution	500–5,000	2,000–20,000	2–20
IV Relatively non-toxic	Caution	5,000+	20,000+	20+

TABLE 2 Organophosphate (OP) identifiers. Data were obtained from comparison of different databases (INCHEM, PubChem, EU Pesticides, and Plant Protection Databases https://www.cabi.org/publishing-products/plant-protection-database/). Water solubility differs according to temperature, while half-life is according to the pH and temperature (short: days–weeks; long: >1 month). Tolerance limits vary by nations and vegetable species.

OP name	Water solubility	Half-life	Tolerance limit om crops (ppm)	Rat oral LC_{50} (mg/kg)	ADI Man/kg/day	Toxic class
Diazinon	40 mg/L 20 °C	Short	0.1–0.005	300–400	0.002	II
Chlorpyrifos	2 mg/L 20 °C	7–120 days	0.1	35–160	0.1	II
Chlorpyrifos-methyl	4 mg/L 20 °C	Short	2.5–0.05	3,000	0.01	III
Fenitrothion	14 mg/L 30 °C	Long	0.2–0.05	250	0.01	IV
Monocrotophos	1 kg/L 30 °C	Long	0.05	8	0.0006	I
Pirimiphos-methyl	5 mg/L 30 °C	Short	0.5–4	2050	0.01	II
Phenthoate	5 mg/L 30 °C	Long	0.05–0.5	300–400	0.003	II

TABLE 3 Carbamate (CB) identifiers. Data were obtained from comparison of different databases (INCHEM, PubChem, EU Pesticides, and Plant Protection Databases https://www.cabi.org/publishing-products/plant-protection-database/). Water solubility differs according to temperature, while half-life is according to pH and temperature (short: days–weeks; long: >1 month). Tolerance limits vary among nations and the vegetable species. n.a., not available.

CB name	Water solubility	Half-life	Tolerance limit om crops (ppm)	Rat oral LC_{50} (mg/kg)	ADI Man/kg/day	Toxic class
Methiocarb	27 mg/L 20 °C	35 days pH 7; 6 h pH 9	NT	0.00025	0.00025	III
Aldicarb	9 mg/L 20 °C	Long	Banned	0.001–0.1	0.005	I
Pirimicarb	2.7 mg/L 25 °C	Short	0.1	100–200	0.02	II
Cartap	High	Short	0.13	325	n.a.	II
Carbaryl	120 mg/L 30 °C	7–28 days	3–75	314	0.01	IV

i. Acute toxicity: resulting from a single, short-term exposure to high concentrations, typically affecting operators and farmers through accidental incidents.
ii. Sub-chronic toxicity: resulting from repeated exposure over weeks or months, usually at low doses during routing handling activities.
iii. Chronic toxicity: resulting from repeated exposure over months or years, affecting bystanders and consumers in polluted areas.

All types of exposure may impact both environmental organisms and humans.

2.6 Human

2.6.1 Acute and chronic intoxication

Pesticide intoxication is one of the most common causes of poisoning worldwide, with approximately one million cases and several hundred thousand deaths reported annually (Pandit et al., 2011). Acute intoxication may result from intentional ingestion or accidental release, as in the 1997 Rhine River spill, which caused widespread mortality in aquatic fauna (Giger, 2007). Both OPs and CBs inhibit ChEs, leading to excess ACh at cholinergic receptors and producing nicotinic (e.g., paralysis and headache) and muscarinic (e.g., respiratory distress, cramps, lacrimation, and potentially death) symptoms. Although early treatment can reverse symptoms, delayed effects may occur as thiophosphate OPs (e.g., chlorpyrifos, diazinon, and parathion) are metabolized to oxon derivatives that are even more potent cholinesterase inhibitors.

Chronic intoxication results from long-term exposure to low pesticide doses. Because residues persist in air and food, exposure is widespread; for example, OP residues up to 0.3 mg/kg were detected in leafy vegetables in 1997 (Morale et al., 1998). Assessing chronic effects is difficult due to low-level mixed exposures and long latency periods, with smoking acting as a confounder through nicotinic receptor stimulation. Bioaccumulation in lipids and cell membranes may contribute to behavioral and neurodegenerative disorders, including an increased incidence of Parkinsonism in areas with intensive pesticide use (Chin-Chan et al., 2015). Respiratory effects include the exacerbation of asthma via vagally mediated bronchoconstriction (Fryer et al., 2004), particularly in smokers (Cloëz-Tayarani and Changeux, 2007). CBs such as carbaryl have also been associated with reproductive toxicity (Kistemaker and Gosens, 2015).

Occupational poisoning rates have reached 18.2 cases per 100,000 agricultural workers and 7.4 per million schoolchildren in developed countries (Thundiyil et al., 2008). Domestic exposures are often underdiagnosed due to nonspecific symptoms. Large cohort studies report an increased risk of hormonally related cancers among spouses of OP users (Lerro et al., 2015) and an association between parental pesticide exposure and childhood leukemia (Clavel et al., 1996). Inheritable alterations in AChE-regulating genes have been proposed as a potential mechanism in tumorigenesis (Soreq and Zakut, 1990). Conversely, some CBs (Baygon, carbaryl, and carbofuran) have been investigated for anticancer properties due to their pro-apoptotic effects in carcinoma cells (Amanullah and Hari, 2011).

2.6.2 Neurological effects

Overall, OPs and CBs can pass through and disrupt the brain–blood barrier (Sinha and Shukla, 2003; Ravid et al., 2018). This allows them to directly affect the central nervous system and interfere with neurotransmitter receptors. Different studies have linked exposure of several CBs and CPs with neurological diseases in humans (Kamel and Hoppin, 2004; Simon-Delso et al., 2015). For instance, maternal exposure to pesticides has been associated with neurobehavioral deficits in newborns (Zhang et al., 2014) as well as impaired memory, reduced psychomotor speed, and mood disturbance in adults (McCauley et al., 2006). In addition, the involvement of OPs in new variant transmissible spongiform encephalopathy has been reported (Purdey, 1994), and OP exposure has been associated with Parkinson's disease (Moretto and Colosio, 2011).

OPs are suspected of contributing to neurological disorders and behavioral disturbance, including ADHD in humans (Slotkin et al., 2007; Bouchard et al., 2010) and even in other models, such as rats (Icenogle et al., 2004). In particular, high levels of DMAP, a metabolite of thionophosphates, have been detected in children and adolescents, supporting a possible relationship between OP exposure and ADHD.

2.6.3 Effects on reproduction and development

Although anti-ChE pesticides have been used as substitutes for endocrine-disrupting PCBs, some (i.e., fenitrothion) may also have antiandrogenic effects (Tamura et al., 2001; 2003). Exposure to OPs and CBs during embryogenesis can impair male reproductive development, leading to reduced sperm production and/or fertilizing capability (Sikka and Gurbuz, 2006), which may contribute to population decline in sensitive wildlife such as amphibians, birds, and reptiles.

Neurotoxic pesticides can cross the placenta and interfere with embryonic development, as demonstrated in both invertebrate and vertebrate models (Falugi et al., 2011; Aluigi et al., 2010a; Aluigi et al., 2010b). Numerous clinical reports describe severe congenital anomalies following occupational or accidental OP exposure during pregnancy (Midtling et al., 1985), and long-lasting depletion AChE and ChE activity has been documented in exposed individuals (Rakonczay and Papp, 2001).

Embryos are extremely sensitive to exposure to neurotoxic pesticides because embryonic development is a multiphasic event, driven by fast ion changes which, in turn, are modulated by the cholinergic system. Molecules related to the cholinergic system are involved in almost all events driving development since sperm–egg interaction, early cleavages up to cell and tissue differentiation, as well as morphogenesis (Falugi and Aluigi, 2012). The earliest developmental stages are the most sensitive, especially in mammals, where inductive signals determine future body structures (Layer and Willbold, 1993; Leitch and Smith, 2013). Later stages are comparatively less vulnerable because developmental potential is more restricted.

2.7 Effect assessment using bioassays

A bioassay or biological standardization is a scientific experiment conducted to measure the effects of a substance on a

living organism or part of it. An insecticide bioassay is any quantitative procedure to determine the relationship between the amount (i.e., dose or concentration) of an insecticide and the resulting biological response (Paramasivam, 2017).

According to Regulation (EU) n. 1107/2009 on pesticide risk assessment, the use of non-animal testing methods and other risk assessment strategies is encouraged. Animal testing should be minimized since it causes pain and suffering, besides being costly and time-consuming. Tests on vertebrates should be undertaken as a last resort, in line with Council Directive 86/609/EEC on the protection of animals used for scientific purposes. Over the past two decades, several European bioassays have been standardized and validated by the EU JRC (reference laboratory for alternatives to animal testing) in accordance with the 3R principle. The latter requires that the use of vertebrates should be reduced in number, refined in execution, and replaced by other models. Thus, *in vitro* screening using biological assays offers an alternative to animal models, being biologically relevant to human physiology (Shukla et al., 2010). In addition, bioethical models such as invertebrates represent good alternatives to evaluate the toxicity of neurotoxic pesticides, the results of which—obtained through bioassays—can be transferred to human and environmental health. Unlike *in vitro* systems, they offer the advantage of studying toxicity within integrated and complex biological systems, encompassing interactions among cells, tissues, and organs.

Bioassays aim at assessing the presence/amount in the environment and eventually in crops, food, and working areas and the drug's effects on organisms. Compared to *in vitro* models, bioassays are based on the exposure of the organism/tissues/cells chosen as models to neurotoxic compounds for a defined time and their ability to detect and quantify measurable biological responses. These responses must then be compared with negative controls (unexposed models).

According to ECVAM (European Centre for the Validation of Alternative Methods), bioassays should be fast, reliable, high-throughput, cost-effective and capable of providing the information required under the REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) and Plant Protection Products (PPP) regulations. Moreover, under the REACH framework, the suite of tests required to assess the admissibility of a substance must ascertain:

- acute toxicity;
- chronic toxicity;
- neurotoxicity;
- genotoxicity;
- mutagenicity;
- carcinogenicity;
- embryotoxicity.

Acute and chronic toxicity consist in any reportable toxic effects in the model after a short exposure to any pesticides. Regarding neurotoxicity and genotoxicity, the investigated biomarkers should be cell proliferation, synaptogenesis, cell death, electrical activity, gene or neurotransmitter expression, and their biosynthetic and lithic enzymes. Conversely, the biomarkers for mutagenicity should be represented by chromosomic alteration, including the presence of micronuclei and apoptosis.

For carcinogenicity, epidemiological studies should be performed. For embryotoxicity, bioassays should be carried out using standardized tests on the development of sensitive models, with first endpoint teratogenesis and several biochemical and biophysical response endpoints.

2.7.1 Selection method

An extensive search was conducted to identify the most reliable bioethical alternative to human models used in toxicological testing. The screening was carried out across multiple platforms between April and May 2025, with the primary source being the publisher-independent citation database Web of Science. Further search tools, such as professional networks and knowledge of authors, Google search, and websites were used to complement the findings. Within Web of Science, the "All databases" collection (coverage: 1900–2025) was queried in English language. Since the term "bioethic model" yielded fewer than five results, we instead used "toxicologic model" and "organophosphate" or "carbamate". To account for heterogeneous functional morphemes, we applied a regular expression syntax (e.g. "organophosphate" to include both singular and plural forms). This search retrieved 277 records to OPs and 125 related to CBs in Web of Science. These results, together with additional sources identified through web searches, were then reviewed to assess their relevance and contribution to the use of bioethical models in toxicology.

2.7.2 Bioethical models

The most reliable models for assessing the effects of CBs and OPs are represented by organisms or part of them that possess a set of cholinergic molecules (Ach), choline acetyltransferase (ChAT), AChE, BuChE, and ACh receptors. Moreover, the requested information is directly correlated with the complexity of the models (Figure 3).

The passage of neurotoxic pesticides across the blood–brain barrier is species-dependent; moreover, ChE enzymes exist in different forms, from globular monomer and dimer to heavy

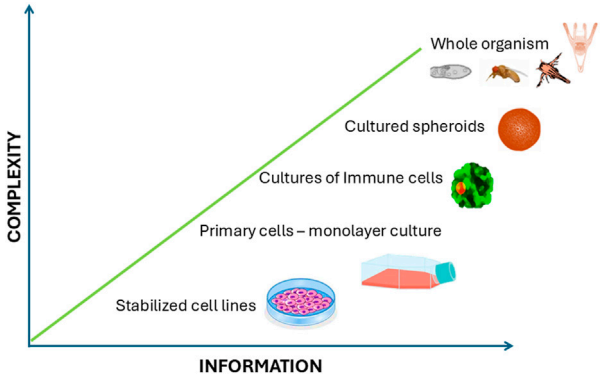

FIGURE 3
Alternative models for neurotoxic pesticide bioassays, including stabilized cell lines, primary cells, cultures of immune cells, cultured spheroids, and whole organisms (e.g., echinoderms, crustaceans, and fruit flies).

asymmetric forms with collagen-like tails (Bon et al., 1979; Masssoulie et al., 1980; 1984). These forms have different enzymatic powers and can quickly convert from one to another when the cell status requests different activity. ChE polymorphisms exist among different animal models, different cells inside the same organism, and even among different status in the same model (De La Porte et al., 1984). Thus, the responsiveness of ChE molecules to neurotoxic pesticides varies among the models. For instance, AChE extracted from electric eels is more resistant to different OP compounds (diazinon, malathion, phenthoate, and chlorpyrifos) than AChE and BuChE from the mammalian brain (Falugi et al., 2011).

The main models used so far to assess human and environmental health due to CB and OP insecticides are:

1. stabilized cell lines (i.e. NT2 and glioma cells);
2. primary cell cultures of nervous cells;
3. Blood cells and cultures of immune cells of invertebrates;
4. cultured spheroids;
5. whole organisms.

The reviewed available literature on the effects of neurotoxic compounds on these different models is now described.

2.8 Stabilized cell lines

Bioassays using these cells were performed to evaluate cell death, cell proliferation, detoxicant enzymes, viability, apoptosis, and mutagenesis. The main endpoints investigated were viability, apoptosis, micronuclei induction, neural differentiation, synaptogenesis, and the expression of cholinergic molecules.

Glioma cells exposed to the OP diazinon at concentrations ranging from 0.002 ng/L to 200 ng/L (concentrations found in mother and children blood and cord samples in agricultural sites; Huen et al., 2012) showed significant effect on micronuclei occurrence in a dose-dependent manner. AChE activity was significantly inhibited by 200 ng/L diazinon (corresponding to approximately 0.6 x 10^{-9} M). In addition, molecules involved in cholinergic activity (i.e. ChAT) and fibronectin showed dramatic variation only in C6 cells exposed to this last concentration. Taken together, these findings translated to the *in vivo* functions of glial cells indicate that exposure to doses that are non-toxic in intact organisms may weaken brain defense and the functions of glial cells through AChE-mediated mechanisms (Falugi et al., 2015).

The NTera2/D1 (NT2) cell line expresses a whole set of molecules related to the cholinergic neurotransmission system, including active AChE. Diazinon at concentrations ranging from 10^{-4} to 10^{-5} M showed a time-dependent enhancement of apoptosis. When exposed at 10^{-6} M, diazinon showed higher cell viability than control samples up to 72 h, followed by a decreasing phase, suggesting that behavior is due to a dynamic balance between activated and blocked ACh receptors that, in turn, trigger electrical events and caspase cascade (Aluigi et al., 2010b).

These findings on stabilized cell lines highlight the importance of investigating apoptosis due to neurotoxic pesticide exposure. Apoptosis represents a crucial biological endpoint in developmental and physiological processes, acting as the transition from cell proliferation to differentiation (Resende and Adhikari, 2009; Hollville et al., 2019). The cholinergic system may play a significant role in regulating programmed cell death (Zakeri et al., 2015). The literature reported above on glioma cells suggests that cholinergic pesticides, particularly OPs such as diazinon, interfere with apoptosis through the overstimulation of ACh receptors. This hyperactivation impairs apoptotic signaling, disrupting the morpho-functional differentiation of structures like the central nervous system, leading to synaptic and morphological redundancy (Caughlan et al., 2004).

2.9 Primary cell cultures of nervous cells

The primary cell culture of nervous cells may provide information on the effects of neurotoxic pesticides including on cell proliferation, cell migration, synaptogenesis, cell death, the production of neurotransmitters and receptors, synaptogenesis, and electrical activity. The main endpoints for mesenchymal stem cells are represented by cell viability and osteogenic differentiation, while those for human leucocytes are ChE expression and activity and leukemia-inhibiting factor (LIF) expression.

The OP chlorpyrifos and the CB carbofuran have no effect on mesenchymal stem cell survival or proliferation up to micromolar concentration; however, limited differentiation capacity was observed when osteogenic differentiation was inhibited (Hoogduijn et al., 2006).

Exposure of leucocytes demonstrated sensitivity to both OP and CB insecticides; specifically, ChE activities were strongly inhibited in mononucleate cells (i.e. lymphocytes and monocytes) (Nejatifar et al., 2022).

2.10 Cultures of immune cells

CBs and OPs are associated with alterations in the immune response (Sharma, 2006). Cultures of immune cells of invertebrates (such as echinoderms and mollusks) represent a population of different cells coworking with different tasks for organism defense (de Almeida Roque et al., 2023). These cells have been used to assess the impact of neurotoxic compounds on the environment. For instance, coelomocytes—a mixed population of immune cells—float in the coelom of adult echinoderms, responding to stress, including that induced by OPs, by impairing AChE activity (Angelini et al., 2003; Falugi et al., 2011; Falugi and Aluigi, 2012).

Hemocytes—cells in charge of the immune response in mollusks—are physiologically and genetically affected by the presence of pesticides, including OPs (Burgos-Aceves and Faggio, 2017). In this regard, OPs decreased the number of hemocytes by reducing cell viability (Ma et al., 2025). The MOA is mainly based on the inhibition of the AChE system, as reported by Dondero et al. (2011), showing altered AChE expression in mussel coelomocytes after exposure to chlorpyrifos.

2.11 Spheroids of neural cells

Three-dimensional (3D) cultured spheroids of neural cells are *in vitro* 3D aggregates of neural cells that mimic the structure and function of brain tissue. Spheroids or 3D spheroid models have been

affected by neurotoxic pesticides in terms of impairing neural development, leading to permanent deficits in the architecture of the nervous system. Thus, the development of nervous structures, the histotypic arrangement of cell layers inside a 3D structure, synaptogenesis, electrical changes, the expression of cholinergic molecules, and the expression of tissue-specific molecules represent the main endpoints to be investigated. Reaggregated spheres from dissociated retinal cells of E6 chick embryos exposed to the OP diazinon at 20–120 μM induced AChE activity reduction, inhibited the differentiation of inner plexiform layer areas, and enhanced apoptosis (Paraoanu et al., 2006). Similar results were obtained after exposure to several CBs (El Sayed et al., 2008).

2.12 Whole organisms

2.12.1 Monocellular organisms

Monocellular organisms have been widely used to assess the impact of neurotoxic pesticides. Among them, protists have been identified as promising models for environmental analysis because they are able to respond to stimuli more quickly than bacteria or yeasts (Hussain et al., 2008). Protists mainly used to assess the impact of neurotoxic compounds, including OPs and CBs, are the marine *Euplotes crassus* (Trielli et al., 2007) and *Paramecium primaurelia* (Falugi et al., 2002a) and the land *Colpoda inflata* (Trielli et al., 2006) and *Dictyostelium discoideum* (De la Porte et al., 1984). Overall, ciliary activity and mating behavior were the most commonly analyzed endpoints.

In *D. discoideum*, exposure to Basudin (20% diazinon formulate) inhibited PrChE activity in a dose-dependent manner in the range of 10^{-1} to 10^{-7} M (60% at 10^{-4} M) without any significant effect on AChE activity. PrChE activity was slightly inhibited by 10^{-5} M eserine and reduced significantly both by 10^{-5} M iso-OMPA and BW284C51; it is classically used to discriminate the different ChE molecular forms. The effects on cell morphology, cell density, and differentiation were evaluated in *D. discoideum* exposed to 10^{-4} M Basudin for 3 days (Falugi et al., 2002a). Such long-term exposure revealed that Basudin-exposed samples showed anomalies in cell morphology and ChE activity inhibition, suggesting that OPs affect the expression of molecules related to cell-to-cell communication.

2.12.2 Metazoans

When bioassays target metazoans, according to the 3R recommendations, exposures are performed at early developmental stages. This assures that the animals are treated before the onset of pain perception; moreover, development supplies the possibility of following chronic exposures in time. Development is a multiphasic event where the different phases rise one from another. Thus, small anomalies not perceived at early stages are subsequently amplified. Moreover, as different developmental events occur at each stage, the analysis of developmental effects allows coupling the toxicity to the mechanisms affected. Lastly, developmental anomalies are more evident earlier at the exposure stage because the morphogenetic fields and developmental potential are restricted along the differentiation, to be reduced to a single expression in adult organisms.

Among metazoans, the following invertebrates have been widely used to assess the toxicity of CBs and OPs: fruit fly (*D. melanogaster),* brine shrimp (*Artemia* sp.), and different species of sea urchins (*Paracentrotus lividus*, *Lytechinus pictus*, and *Strongylocentrotus purpuratus*). The literature on these models is discussed below.

2.12.2.1 Fruit fly

OP and CB insecticides irreversibly inhibit AChE, causing the death of the insects. Thus, homogenates of fruit fly brain or whole adult organisms have been used to test pesticides, as this fly is one of the target species of neurotoxic pesticides (i.e. diazinon, malathion, and carbofuran). Generally, this model is used to test resistance to neurotoxic pesticides, as demonstrated by analyzing AChE activity expression and structure in several fruit fly species (i.e. *D. melanogaster* and *Bactrocera dorsalis;* Fournier et al., 1992; Mutero et al., 1994; Menozzi et al., 2004; Hsu et al., 2008; Vargas et al., 2015). Thus, Li et al. (2013) reported AChE inhibition in *D. melanogaster* exposed to either CBs or OPs. In addition, some CB compounds affected the survival of *D. melanogaster* adults and progeny, inducing toxicity (Marchal-Segault et al., 1985).

Considering the high sensitivity of AChE from the fruit fly *D. melanogaster* to OPs and CBs, engineered biosensors have been constructed for the rapid detection of CB and OP compounds (i.e. paraoxon and malaoxon; Villatte et al., 1998; Bachmann et al., 2000; Xu et al., 2007).

2.12.2.2 Brine shrimp

The brine shrimp of genus *Artemia* was one of the first invertebrate organisms, together with another crustacean *Daphnia magna*, to be used in environmental bioassays for testing the toxicity of pesticides (Stevenson, 1961; Baek et al., 2016; Jawahar et al., 2018); it is still in wide use (Albarano et al., 2022). As in most arthropods, *Artemia* sp. has a largely cholinergic nervous system, strongly marked by the histochemical reaction of AChE (Raineri and Falugi, 1983). Nevertheless, it is a low sensitivity model because it is very resistant to environmental conditions. Jawahar et al. (2018) found high toxicity levels after short exposure to the two OPs malathion and glyphosate (LC_{50}: 58.3 ppm for malathion; 0.019 ppm for glyphosate).

Bioassays on brine shrimps aim to verify the possibility of this model responding to low-dose toxicity, such as that possible in marine environments (Hoogduijn et al., 2006). Among the bioassays, hatching success rate, early development, mortality, and enzyme activity impairment are the most used endpoints for CBs and OPs (Barahona and Sánchez-Fortún, 1999; Varó et al., 2002; Bustos-Obregon and Vargas, 2010). Thus, the resistance cysts of *Artemia salina* at G II instar rehydrated in water containing CBs or OPs at concentrations of 10–11 to 10–5 M up to 192 h affected morpho-functional and biochemical parameters, such as hatching speed, larval anomalies, survival, and ChE activity (Gambardella et al., 2018). Regarding hatching, the OP chlorpyrifos oxon and the CB carbaryl caused a significant dose-dependent decrease in hatching speed, followed by high percentages of larval death, while chlorpyrifos and diazinon mainly caused an irregular pattern of hatching. OPs strongly impact even brine shrimp early developmental stages; for instance, diazinon affected the survival and metabolic rate of nauplii and larvae up to 48 h exposure (Gartenstein et al., 2006).

Since the cholinergic system has a key role in regulating programmed cell death, disturbances in signaling may underlie embryonic malformations and growth defects in invertebrates during early developmental stages, as reported for brine shrimps.

2.12.2.3 Sea urchins

Sea urchin (*P. lividus*, *L. pictus*, *S. purpuratus*, etc.) have long been used for assessing the toxicity of chemical compounds, radiation, and other physical–chemical stressors. For ease of sampling, rearing in the laboratory, and overall ease of obtaining gametes and embryos in high quantity, they were developed synchronously inside transparent envelopes and maintained body transparency up to late stages. This allowed the immediate perception of anomalies and statistical elaboration of data as well as the possibility of following the late effects of chronic exposures. The presence of the full set of molecules associated with the cholinergic cell-to-cell communication system from the interaction between gametes (Aluigi et al., 2008), early development (Ohta et al., 2009), to the morphogenetic movements and differentiation of nervous-like structures (Falugi et al., 2002b; 2008; Pesando et al., 2003) makes the echinoderm an optimal model for detecting the effects of neurotoxic pesticides.

According to the sequencing of its genome (2006) and high affinity with the vertebrates and humans, the sea urchin was proposed as a biosensor to test the effects of various pesticides and organic compounds in early development (Buznikov et al., 2003). Moreover, the AChE of *P. lividus* has been considered a biomarker of environmental contamination (Cunha et al., 2005) and was proposed for the study of Alzheimer's disease (Buznikov et al., 2008) and suggested as a biosensor (Buznikov et al., 2003).

Recently, Morroni et al. (2016), Morroni et al. (2018) identified a new method of establishing integrated toxicity (integrated toxicity index) by combining the number of anomalous embryos and the severity of the anomalies. Likewise, Gambardella et al. (2021) proposed a new index of contaminant impact based on morphological anomalies of early-stage sea urchins due to different contaminants, including neurotoxic compounds such as OPs and CBs, to assess the health of coastal waters.

The main endpoints for assessing the toxicity of OPs and CBs in sea urchins are spermiotoxicity (sperm capability to fertilize eggs after pesticide exposure), embryotoxicity, developmental and skeletal anomalies, biochemical activities (i.e. AChE, BuChE activity), mutagenesis—detected in term of micronuclei and apoptosis—and genotoxicity (Aluigi et al., 2005; 2010a; Pesando et al., 2003; Falugi et al., 2008; Deidda et al., 2021; Gambardella et al., 2021). Overall, the balance between cell survival and programmed cell death in sea urchins is significantly disrupted by cholinergic pesticide exposure, with implications for neurodevelopment and potential links to neurodegenerative outcomes.

Skeletal anomalies in sea urchins should be investigated to assess the effects of neurotoxic compounds. Indeed, a deficit in skeletal spiculae mineralization in sea urchin larvae after OP Basudin exposure has been demonstrated (Morale et al., 1998), paving the way for possible implications in osteoporosis (Compston et al., 1999), and long-term effects were associated with cardiovascular diseases (Hung et al., 2015).

3 Discussion

This review has focused on two ECs with anti-cholinesterase properties. Thus, the MOA of OPs and CBs, which makes them noxious to human and environmental health, is described. The MOA of these neurotoxic pesticides is mainly the inhibition of AChE activity (IRAC, 2020) that affects neuromuscular functions but also embryogenesis and new generations, as reported for humans and other models. Besides the modulation of impulse transmission at synapses, AChE has several non-neuromuscular functions wherever cell-to-cell communication is involved (Falugi et al., 2012), from sperm–egg interactions to the transmission of inductive messages, morphogenetic movement guidance, and a few differentiative events. In addition, secondary targets of OPs are represented by pseudo-cholinesterase and nicotinic and muscarinic receptors (Aluigi et al., 2005; 2010a; 2010b) involved in bronchial and cardiac regulation, as well as in the regulation of endocrine glands responses—being suspected endocrine disrupters (Sohoni et al., 2001; Tamura et al., 2001).

Although in recent years the release of pesticides into the environment to ensure intensive crops has slightly decreased or at least has been carried out in a targeted manner, the presence of pesticides in environmental compartments is still high and almost ubiquitous. Currently, research on pesticides is mainly aimed at the development of new substances for which an increasingly specific effect is sought for the species against which they must act and in the evaluation of the effects of these chemicals on other non-target species and humans.

Hazard identification and the harmful effects of OPs and CBs have been reported not only in humans but also in other bioethical models, for which several bioassays are available. Unlike what has been done so far, we suggest a panel of sensitive models and bioassays with different sensitivity to neurotoxic compounds, offering the possibility of testing different endpoints and easy measurable biomarkers that may affect single cells or organisms up to cell death or mortality. Providing this panel of sensitive models and bioassays aims at enhancing the predictivity and risk assessment of human and environmental health.

Author contributions

CG: Conceptualization, Data curation, Formal Analysis, Resources, Supervision, Writing – original draft, Writing – review and editing. PS: Writing – review and editing. OP: Supervision, Writing – original draft, Writing – review and editing. CF: Conceptualization, Data curation, Writing – original draft, Writing – review and editing. FM: Writing – review and editing. MF: Supervision, Writing – review and editing.

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Supplementary material

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