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Chapter 29

Using isotope tracers to elucidate the fate of organic micropollutants in the environment

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29.1 Introduction

Micropollutants are defined as pollutants found in the environment at trace concentrations (ng/L or μg/L) and are of potential toxicological concern [1,2]. The organic pollutants include synthetic and natural compounds such as pesticides, pharmaceuticals, personal care products, endocrine-disrupting chemicals, and other industrial chemicals [3]. Originating from both natural processes and human activities, micropollutants are widely present in various environments, including wastewater from hospitals, industries, and livestock, surface runoffs from agriculture and aquaculture, and leachates from contaminated soils and landfills [1,4]. For example, pesticides like neonicotinoids have been reported to widely contaminate surface water at an average concentration of 0.13 μg/L [5]. A comprehensive study, monitoring 61 pharmaceuticals across 1052 sampling sites along 258 rivers in 104 countries revealed total concentration of up to 70.8 μg/L [6]. The personal care product component triclosan was detected in >40% of samples from 686 sampling sites in 10 countries with a maximum concentration of 28.0 μg/L [7]. Steroid hormones such as estrone and estradiol were detected at concentrations ranging from 0.2 to 147 ng/L in domestic wastewater [4] and 0.2 to 33.9 ng/g in soils and sediments [8]. Industrial chemicals such as plasticizers (e.g., bisphenol A (BPA) and its alternatives [9]), surfactants (e.g., nonylphenol [10]), and fire retardants (e.g., tetrabromobisphenol A, TBBPA [11], polybrominated diphenyl ethers [12], per- and polyfluoroalkyl substances, PFAS [13]) have also been frequently detected at trace level in water, soils, and sediments. Especially, PFAS that contain at least a perfluorinated methyl group (−CF3) or a perfluorinated methylene group (−CF2 −) are raising increasing concern due to its extremely high persistence, mobility, and bio-accumulative properties [14]. As a result, maximum levels of six PFAS were set at 1 − 4 ng/L in the most recently published National Primary Drinking Water Regulation of EPA [15].

Micropollutants pose a significant concern due to their potential toxicity to organisms in various environmental matrices. For instance, pesticides in the environment have been defined as a causal factor in the decline of invertebrates in terrestrial ecosystems, leading to significant impacts on the structure and dynamics of food webs [16]. Hazard quotient assessment has revealed that at 270 out of 1052 river sampling sites the concentrations of at least one of the 61 pharmaceuticals exceeded predicted no-effect concentrations, indicating their potential ecotoxicity risks to algae, daphnia, and fishes [6]. Moreover, micropollutants have been linked to several human diseases, such as endocrine disruption disorders and cancers [17]. For example, steroid hormones can induce reproductive disorders and feminization in organisms, both human and aquatic [17]. Antibiotics, as one of the widely used pharmaceuticals, are a driver of antibiotic-resistant bacteria and antibiotic-resistance genes, which significantly affect the health of humans and other organisms [18] and are estimated to be associated with approximately 4.95 million deaths in 2019 alone [19]. Finally, PFAS can interact with blood proteins and induce various pathological responses, including hepatotoxicity, tumor induction, developmental toxicity, immunotoxicity, neurotoxicity, and endocrine disruption [20].

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Given these facts concerning the occurrence and effects of micropollutants in the environment, research has been conducted to investigate their fate in various matrices. Micropollutants can bind to matrix components (e.g., solids, organic matters), break down into known or unknown transformation products (TPs) via biodegradation, photolysis, or other abiotic processes, bioaccumulate in organisms, and interact with other coexisting environmental pollutants [21,22]. The fate of micropollutants in complex environmental matrices is compound- and condition-specific. Previous studies primarily focused on the removal of parent micropollutants only or further on their transformation into several TPs, due to the trace concentrations of micropollutants, their wide distribution, and interference by other organic compounds during chemical analysis. As a result, mass balances of the target micropollutants were scarcely established when studying biodegradation processes. Moreover, in sediment/soil matrices and when bioaccumulated in organisms, many micropollutants primarily form nonextractable residues (NERs), which are resistant to extraction and are therefore unmeasurable in the matrix as parent compound or its TPs [23,24]. However, limited studies have delved into the formation, composition, stability, and bioavailability of NERs when studying the fate of micropollutants. Apart from NER formation, it is difficult to delineate the contribution of different pathways (e.g., adsorption, biotic and abiotic degradation) and main parameters (e.g., micropollutant-degrading bacteria and their interaction as a community, environmental factors) to the fate and transformation of micropollutants.

Radioactive and stable isotope tracers provide valuable opportunities to investigate the overall fate of micropollutants and reveal the underlying mechanisms. With radioactive-labeled target micropollutants, their turnover into different isolated phases of the matrices, degradation into TPs, formation into NERs, and bioavailability to various organisms could be elucidated [25]. An alternative is the use of stable isotope tracers, which do not have the same laboratory safety and handling restrictions as radioactive-labeled compounds, making them more versatile. Stable isotope tracers can be applied to identify the origin and fate of micropollutants via compound-specific isotope analysis (CSIA), delineate the active members and their roles in microbial communities during micropollutant biodegradation via stable isotope probing (SIP), and even sort out the active members when combined with single-cell Raman spectroscopy (SCRS) [26–28].

In this chapter, we provide a summary of studies using radioactive isotope tracers to investigate the fate of micropollutants in water, soil, sediment, and organisms. Additionally, we summarize studies that revealed the underlying transformation mechanisms of micropollutants in environments using stable isotope tracers. Finally, we also highlighted the challenges and outlook for isotope tracer application for future studies.

29.2 Use of radioactive isotope tracers

Radioactive isotopes, such as ^{14}C-labeled compounds, can be introduced into a variety of environmental samples, including waters, soil, sediment, animals, and plants, to investigate the fate of micropollutants. The separation, extraction, and detection procedures for radioactivity are concisely summarized in Fig. 29.1. The extraction procedure has traditionally been accomplished by solvent extraction methods, including batch solvent shaking extraction, continuous Soxhlet extraction, and improved versions such as the Soxtec extractor [29]. Subsequently, new extraction technologies such as ultrasonication, microwave extraction, supercritical fluid extraction, and accelerated solvent extraction have been employed [25].

29.2.1 Fate of micropollutants in natural and engineering water systems

Concentrations of micropollutants are typically lower than μg/L levels in natural and engineering water systems. Their occurrence is normally accompanied with organic matters or other components in the matrices, which to a great extent interfere with quantitative measurements of the parent micropollutants and their TPs. A variety of lab-scale studies have investigated the fate of micropollutants in waters, but due to analytical challenges, employed concentrations much higher than those found in the environment. Radioactive isotope tracer technique emerges as a powerful tool to test distribution and (bio)degradation of radiolabeled substances at concentrations in the same order of magnitude as expected in water [30]. For example, reversible transformation of ^{14}C-bisphenol A (BPA) was proven to occur at low concentrations under anoxic conditions with the involvement of acetate kinase and hexokinase [31]. This enzymatic transformation mechanism was not observed in their previous study, where a high concentration of BPA was applied to facilitate the detection of possible TPs [32]. This indicates the importance of studies at realistic concentrations.

In the Scientific Guideline of environmental risk assessment of medicinal products for human use published by the European Medicines Agency [33], the biodegradability of the substances and particularly their elimination in wastewater is of high importance. Radioactive isotope tracers offer precise assessment of micropollutant biodegradability. For instance, no mineralization of ^{14}C-ciprofloxacin was observed in an aqueous system, indicating its recalcitrance to biodegradation and transformation [34]. With the application of ^{14}C-labeling, degradability of several antibiotics was tested

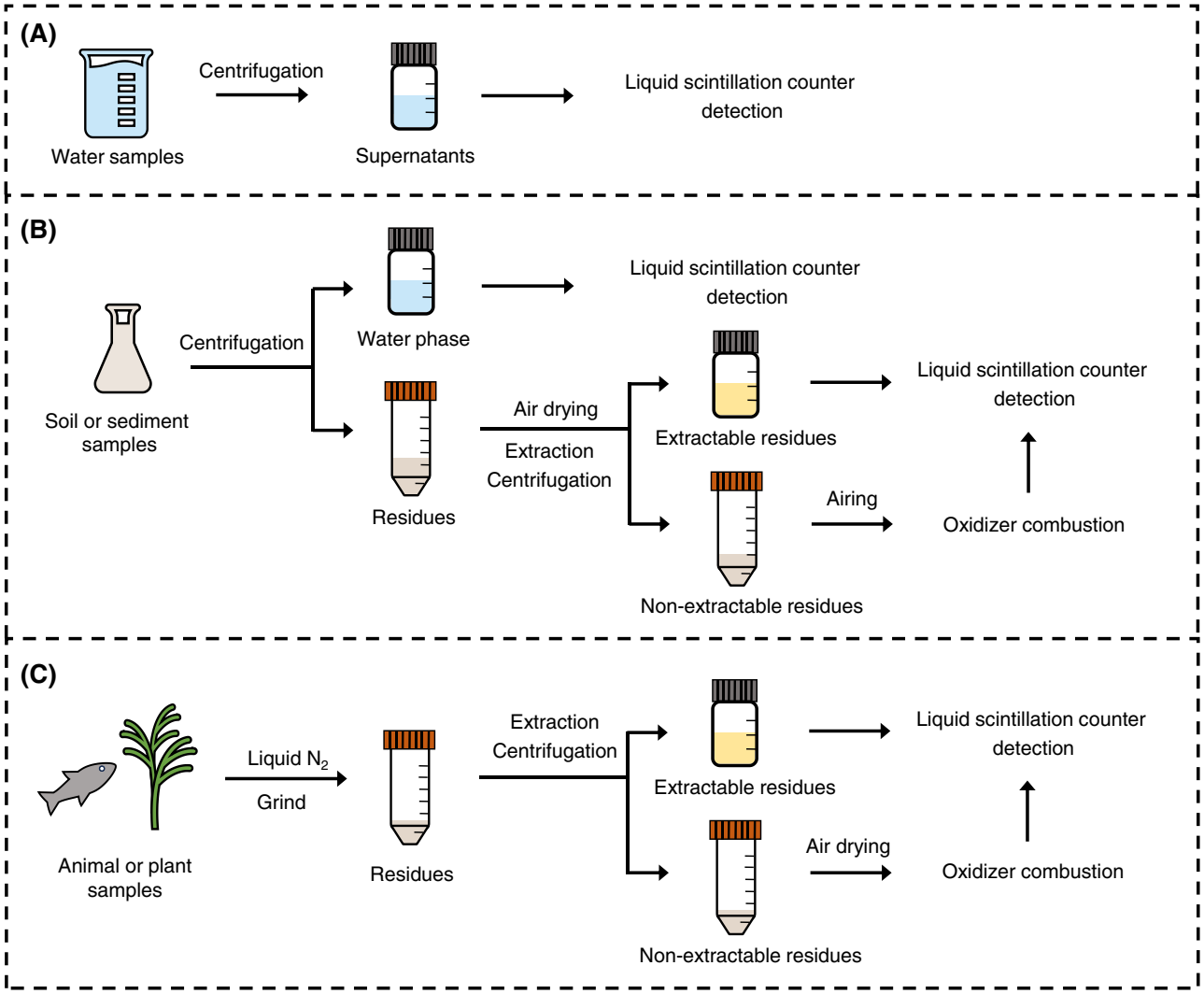

FIGURE 29.1 Detection of radioactivity derived from radioactive-labeled compounds in (A) water, (B) soil/sediment, and (C) animal and plant samples.

in an activated sludge wastewater treatment system, where 25% of benzylpenicillin was mineralized, but ceftriaxone and trimethoprim were not mineralized at all [30]. In a membrane bioreactor system, employed as posttreatment in wastewater treatment, mineralization rate of ^{14}C-labeled micropollutants including ibuprofen, naproxen, and diclofenac could achieve approximately 0.8 ng/mg$_{TSS}$ h with a short lag phase [35].

An advantage of experiments with ^{14}C-labeled micropollutants is its feasibility to differentiate between mineralization and abiotic elimination, e.g., adsorption. For example, in granular-activated carbon biofilms where both adsorption and biodegradation occurred, it is difficult to strictly separate the contributions of these two processes to the overall micropollutant removal. By monitoring ^{14}C activities in the liquid and gas phases, microbial cleavage of ibuprofen, naproxen, diclofenac, and mecoprop was confirmed in granular-activated carbon filters based on the formation of $^{14}CO_2$, whereas removal of carbamazepine was attributed to adsorption [36]. When advanced oxidation processes are combined with adsorption processes, it remains challenging to determine to what extent the micropollutant TPs formed during oxidation can be removed by subsequent adsorption, given that these TPs are typically largely unknown compounds. By determining the adsorption capacity of powdered activated carbon toward the sum of all ^{14}C-labeled TPs of nine pharmaceuticals produced by ozonation, Betsholtz et al. [37] confirmed that adsorption to activated carbon alone is not a viable removal method for a wide range of ozonation TPs.

Another advantage of using radioactive isotope tracers is that the technique allows to close the mass balances of micropollutants and to circumvent analytical difficulties in complex matrices [30,38]. For instance, by closely

monitoring ^{14}C-radiolabelled residues distribution in the solid, liquid, and gas phases, sulfamethoxazole (SMX) was demonstrated to be mainly transformed by heterotrophic and autotrophic biomass in activated sludge independent of redox conditions, with mineralization and sorption remaining below 5% [39]. Moreover, the mass balance of nonylphenol in a lab-scale membrane bioreactor indicated that 42% of the applied radioactivity was recovered in the effluent as TPs of nonylphenol, 21% was removed with the daily excess sludge from the reactor, and 34% was adsorbed in the component parts of the reactor [40]. During treatment by activated sludge, ^{14}C-dicofol organochloride was mainly transformed to dichlorobenzophenone with only 0.1% of mineralization, and 57% of the radioactivity remained adsorbed on the sludge as dichlorobenzophenone [41].

Compared to the extensive use of radioactive isotope tracers in wastewater studies, their application in investigating micropollutant fate in surface water, drinking water, and groundwater has received limited attention. Among these studies, the fate of ciprofloxacin in surface water was confirmed to be strongly influenced by adsorption and photodegradation, with the adsorption capacity significantly affected by the level of particulate organic carbon [42]. By measuring the biodegradability of 2,6-dichlorobenzamide (BAM), Hylling et al. [43] demonstrated a promising hybrid concept using a combination of membrane filtration with BAM-degrading bacteria *Aminobacter* sp. MSH1 augmentation for drinking water treatment. In groundwater treated by full-scale rapid sand filters, the fate of ^{14}C-labeled mecoprop was studied, revealing a 79%−86% removal after 24 hours. Sorption accounted for 11%−15% of removal, and the remaining mecoprop was eliminated by biodegradation, leading to 13%−18% of mineralization [44].

29.2.2 Fate of micropollutants in soil

After entering soil, micropollutants undergo diverse abiotic and biotic turnover processes including degradation, uptake by soil organisms or plants, and formation of NERs [24]. Using radioactive isotope (such as ^{14}C) tracer technology, the fate of various micropollutants has been explored in soils (Fig. 29.2). For example, as the most widely used BFR, TBBPA has been found to dissipate in soils under various conditions (e.g., oxic, anoxic, or flooded), accompanied by mineralization and significant formation of NERs. TBBPA could be degraded into TPs mainly via four interconnected pathways (oxidative skeletal cleavage, *O*-methylation, Type 2 ipso-substitution, and reductive debromination) [45−49]. During the degradation process, up to 11.5% of TBBPA and its TPs were mineralized after 66 days of incubation in flooded soil with adjacent anoxic and oxic layers [49], which was more than that in oxic and anoxic soils (1%−9.2%) [45−49]. Simultaneously, 40%−80.3% of TBBPA and its TPs formed NERs in soil, which is the most significant fate of TBBPA in soils.

Formation of micropollutant-derived NERs largely depends on the soil property, redox conditions, and microbial activity [45−49]. For example, under oxic conditions, TBBPA and its TPs formed higher amounts of NERs in silty clay soil (80.1%) [48] than in sandy soil (66.5%) [55], likely due to greater surface adsorption energy and adsorption sites in silty clay soil [56]. Compared to anoxic conditions, oxic conditions fostered substantial TBBPA-derived NERs, possibly because the presence of oxygen promoted formation of TPs with active chemical properties, which covalently bound to soil organic matters and formed into NERs [55]. In sterilized soils, the abiotic aging processes of TBPPA involved

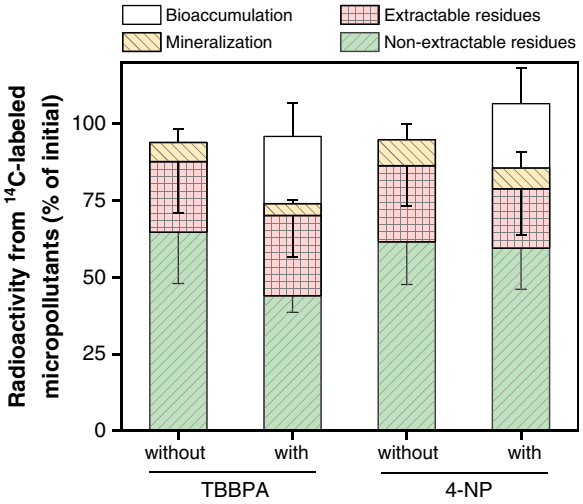

FIGURE 29.2 Radioactivity recovered from ^{14}C-labeled micropollutants incubated with/without earthworms or plants in soils, including bioaccumulation, mineralization, and formation of extractable and nonextractable residues. Tetrabromobisphenol A (TBBPA) and 4-nonylphenol (4-NP) are taken as the examples [45−54].

adsorption on soil matrixes and diffusion into micropores of soil aggregates, which led to formation of sequestered and entrapped NERs (Type 1 NERs) [45]. In comparison, microbial activity significantly enhanced formation of TBBPA-derived NERs and played a vital role in the formation of covalently bound residues and biogenic NERs (Type 2 and 3 NERs) [45]. Similar patterns were also observed in other micropollutants, such as 4-bromodiphenyl ether [57], 4-nonylphenol [50], neonicotinoid [58], phenanthrene [59], and sulfadiazine [60]. In addition, studies also indicated distribution of NERs in fulvic acids, humic acids, and humin of soil based on alkaline solubility of micropollutants. For most of the micropollutants investigated, such as BPA [53,61], bisphenol F (BPF) [62], 4-bromodiphenyl ether [57], 4-nonylphenol [50,52], phenanthrene [59], and TBBPA [45,47,49], the majority of NERs was found in the humin fraction, which contains more organic carbon, kerogen and black carbon particles, potentially offering more adsorption sites for organic contaminants [63,64]. This could be elucidated because of the use of radioactive-labeled compounds and could not have been observed using traditional chemical analyses.

Soil organisms such as earthworms and plants also strongly influenced the fate of micropollutants (Fig. 29.3). Application of earthworms is considered a potential approach for remediating micropollutant-contaminated soil due to their ability to stimulate soil microbial activity and improve soil aeration and nutritional status [47,65]. Micropollutants could be accumulated in earthworms predominantly as NERs [59] and distribution of micropollutants in earthworms was influenced by their living behaviors. Earthworms like *Metaphire guillelmi* [62,66,67] and *Pheretima guillemi* [68] that exhibit geophagous traits showed higher accumulation of micropollutants in their guts. In contrast, *Eisenia fetida* that ingests little soil primarily accumulated micropollutants in their skin [46]. Apart from accumulation of micropollutants, the presence of earthworms inhibited mineralization and affected NER formation of micropollutants by using radioactive isotope tracers. For instance, *M. guillelmi* not only altered the transformation pathways of TBBPA but also inhibited its mineralization from 3.9% to 2.6% [47]. Similar effects were also observed in phenanthrene, where in the presence of *M. guillelmi* the mineralization decreased from 40% to 24.4% [59], and BPF from 26.9% to 12.4% [62]. This phenomenon might be attributed to several factors: (1) accumulation of micropollutants and their TPs in earthworms [51]; (2) reduction in the population of microorganisms degrading micropollutants in soil, considering *M. guillelmi* can efficiently digest soil microbial biomass [69]; (3) decrease in bioavailability of micropollutants owing to their entrapment in soil aggregates formed by the earthworm or their strong interaction with soil mineral particles during earthworm gut passage [59].

The influence of earthworms on NER formation of micropollutants is complex. On the one hand, earthworms could enhance the NER formation by facilitating physiochemical trapping of micropollutants on clay minerals and humus substances, and transformation of micropollutants (e.g., atrazine, a pesticide) [70] into TPs that easily form NERs during gut passage. On the other hand, earthworms also showed inhibition of NER formation by altering the compositions of the micropollutant TPs in soil. Gu et al. [47] have estimated that the presence of *M. guillelmi* could inhibit the formation of the polar TPs of TBBPA, which preferred to be incorporated into soil humic substances via ester linkages to form NERs [55]. Similar decreases in NER formation were also found in phenanthrene from 70.1% to 64.5% [59] and TBBPA from 80.3% to 41.8% [47].

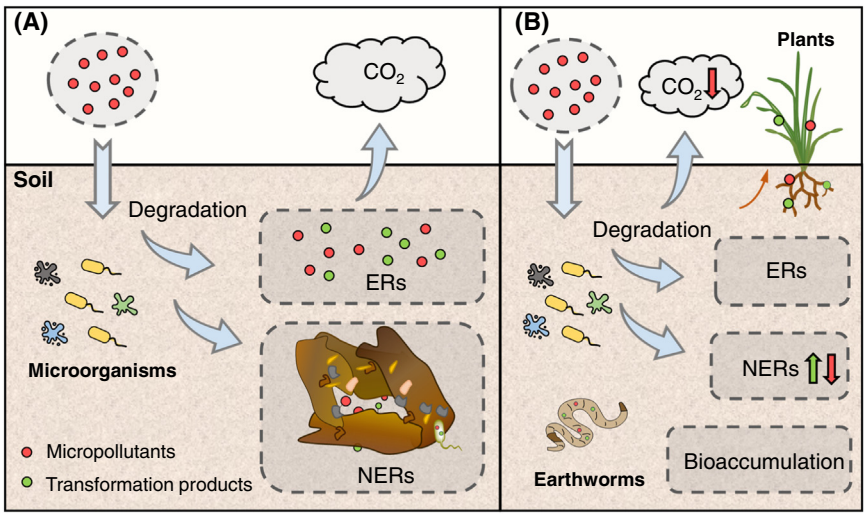

FIGURE 29.3 Schematic diagram of the fate of micropollutants in soils (A) without and (B) with soil organisms including earthworms and plants. Soil organisms could not only accumulate micropollutants but also inhibit their mineralization and increase/decrease NER formation of micropollutants. *NER*, Nonextractable residue.

Radioactive-labeled compounds also showed the effect of plants on the mineralization and NER formation of micropollutants in soil through uptake, phytodegradation, and rhizosphere effects (Fig. 29.3). The paddy rice (*Oryza sativa*) inhibited mineralization of TBBPA [49] but promoted BPA mineralization [61] in a silty clay loam soil. The formation of TBBPA-derived and BPA-derived NERs was also inhibited in the presence of rice (*O. sativa*) [49,61] or reed (*Phragmites australis*) seedlings [49,61], probably attributed to accumulation of micropollutant in the plants or release of NERs due to the rhizosphere effects [49].

Although formation of NERs was considered the main way of soil to detoxify micropollutants [71], alteration of soil properties and activity of soil microorganisms may lead to release of Type 1 and 2 NERs. Previous studies have shown that a significant amount of the TBBPA-derived (9.2%) [48] and phenanthrene-derived NERs (33.9%) [59] could be mineralized after re-incubation in active soil. Liu et al. [45] also demonstrated that 42% of NERs derived from TBBPA and its TPs were released after shifting soil from anoxic to oxic conditions. Degradation capacity of soil microorganisms also affected stability of the NERs formed. With the amendment of TBBPA-degrading *Ochrobactrum* sp. T, 10.9% of TBBPA-derived NERs were released and transformed [72]. As the oxic microsites around the plant roots could alter the redox conditions and microbial community of soil [49], the NERs derived from SMX were released (11%) and further taken up by plants (0.2%) [73]. Similarly, the activity of earthworms also promoted the release of micropollutant-derived NERs [73]. Therefore the formation and potential release of NERs are crucial for investigating the fate and assessing the environmental risks of micropollutants in soil. This shows that radioactive isotope tracers are an appropriate tool to illustrate the formation, fractionation, stability, and bioavailability of micropollutant-derived NERs in soil.

29.2.3 Fate of micropollutants in sediment

Radioactive isotope tracers have been used to study the fate of micropollutants in sediments. Nearly two decades ago, radioactive isotope tracers have been applied to study the sorption and adsorption behavior of tributyltin in estuarine sediments, where tributyltin partitioning was concentration-dependent and influenced by salinity and pH of the sediment [74]. Similarly, the removal of three ^{14}C-labeled pharmaceuticals by both adsorption and degradation was investigated in river water sediment [75]. The results show that ibuprofen and paracetamol displayed a low persistence with half dissipation times (DT_{50}) $\leq$ 20 days. Ibuprofen and its TPs were rapidly biodegraded, while paracetamol formed NERs rapidly and extensively. In contrast, another pharmaceutical diazepam exhibited high persistence with a DT_{90} value exceeding 1 year. Moreover, the influence of sediment organisms on the fate of micropollutants has also been explored. Wang et al. [76] explored the fate of three novel brominated flame retardants (BRFs) in a sediment-water-mudsnail system, where the BRFs adsorbed onto the sediment scarcely remained in the water but were accumulated by the mudsnail at 2.0−22 mg/kg dry weight. Additionally, the average enrichment of BFRs in viscera was about three times higher than in pleopod, and the parent mudsnails were able to transfer the accumulated BFRs to their offspring.

Using the radioactive isotope tracers, mass balance of micropollutants in water sediment systems was also determined to evaluate their fate and distribution (Fig. 29.4). Holzmann et al. [80] have investigated the fate of several ionic

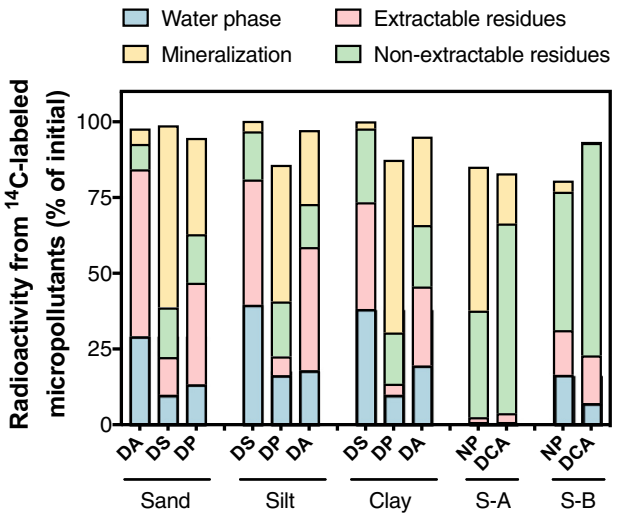

FIGURE 29.4 Turnover of ^{14}C-labeled micropollutants in sediment-water systems. DA, DS, DP, NP, and DCA refer to 4-n-dodecylbenzyltrimethylammonium chloride, 4-n-dodecylbenzenesulfonic acid sodium salt, 4-n-dodecylphenol, 4-nitrophenol, and 3,4-dichloroaniline. S-A and S-B represent sandy sediment and sediment with high clay/silt and organic carbon content, respectively. The data shown are mean values obtained from previous studies [77−80].

micropollutants with similar structure but different charges in sediments. Their findings demonstrated that positively charged 4-n-dodecylbenzyltrimethylammonium chloride (DA) exhibited higher persistence in sediment compared to the anionic 4-n-dodecylbenzenesulfonic acid sodium salt (DS) and nonionic 4-n-dodecylphenol (DP). The high persistence of DA was due to its strong binding affinity of DA to the sediment matrix, leading to a reduced bioavailability and thus the mineralization capacity. Additionally, differently sized particle fractions and texture of the sediment influenced NER formation of DA and DP, whereas anionic DS remained unaffected. Higher portions of NERs derived from DA and DP were formed in the clay fraction of sediment compared to the sand and silt fractions [79]. Similarly, mass balance analysis of two pesticide-derived TPs demonstrated that 3,4-dichloroaniline was stable in sediment-water systems due to adsorption while 4-nitrophenol showed low persistence and accumulation [77]. NER formation of 3,4-dichloroaniline was mainly attached to the insoluble humin fraction (54%), whereas minor amounts were associated with fulvic (13%) and humic acids (6%) after 28 days of incubation [78]. Both of the two micropollutants exhibited less mineralization but favored NER formation in sediment with high clay/silt and organic carbon content compared to sandy sediments [78]. In addition, nonylphenol isomer was found to distribute into sediment immediately following application in water, with up to 41% detected in sediment after 14 days of incubation. However, this micropollutant was resistant to biodegradation, with only 4.2% loss attributed to mineralization and volatile TPs formation after 28 days [81].

In comparison to sediments from ponds, rivers, and lakes, fewer studies have examined the fate of micropollutants in groundwater sediments. In the study of Barber et al. [82], sediments from the transition and contaminated zones of groundwater were collected and biodegradability of the two micropollutants was evaluated under oxic conditions. The findings illustrated that 4-nonylphenol was more rapidly biodegraded (30% − 60% of mineralization in 13 days) than 17β-estradiol (20% − 90% of mineralization in 54 days). Interestingly, there was little variation in the biodegradability of the target micropollutants across sediment sampling sites. Further studies are needed to investigate the fate of micropollutants in groundwater sediments by employing radioactive isotope tracers in the future.

29.3 Use of stable isotope tracers

29.3.1 Compound-specific isotope analysis technique

Degradation of micropollutants involves both biotic and/or abiotic transformations, including bacterial enzymatic and/or (photo)chemical reactions. Typically, the lighter isotopes at specific positions within a compound react faster than the heavier isotopes. By determining the slight isotope fractionation effects that occur during the degradation process, CSIA provides a unique opportunity to identify the origin and fate of micropollutants in the environment [83,84]. Distinct from conventional analytical methods that rely on measuring parent compounds and their TPs, CSIA can provide direct evidence of the extent of degradation based on the isotopic fractionation information, even without detection of known or unknown TPs [85,86]. To date, CISA has been applied to investigate the fate of micropollutants including pesticides, various industrial compounds, pharmaceuticals and personal care products, nitroaromatic compounds, and organic explosives in diverse environments such as rivers, lakes, groundwater, wastewater, and contaminated field sites [28,84,86,87].

Environmental micropollutants are present at trace-level concentrations and can coexist with other organic components. The sensitivity of CSIA is often lower than the conventional concentration analysis. To facilitate CSIA application for micropollutants, innovative sampling, preconcentration, and clean-up techniques have been developed [28]. The preconcentration and clean-up methods include purge and trap, solid-phase microextraction, and solid-phase extraction (Fig. 29.5), where the possibility of isotope fractionation during sorption, desorption, and phase transfer needs to be systematically validated [27]. Gas chromatography-isotope ratio mass spectrometry is the most common detection method for CSIA, suitable for volatile or semivolatile compounds, though derivatization may be necessary [27,83]. Alternatively, liquid chromatography coupled to IRMS (Fig. 29.5) provides an option for polar and nonvolatile compounds [83].

To address changes in molecular stable isotopic signatures caused by nonreacting atoms, position-specific isotope analysis (PSIA) has been developed to study the intramolecular isotopic variations [89]. PSIA is considered as the next level analysis for mechanistic isotopic fractionation studies of organic pollutants including micropollutants [90]. Additionally, the concept of 2D- or multielement CSIA has been developed to avoid the additional isotope fractionation effects, e.g., masking effects derived from the rate-limiting steps preceding bond cleavage and superimposed isotope effects from multiple chemical reactions [91,92].

First, in the context of CSIA application in exploring the fate of micropollutants, the conservative isotope makes CSIA pivotal for determining the source of micropollutants. For example, the most likely suppliers of benzotriazoles

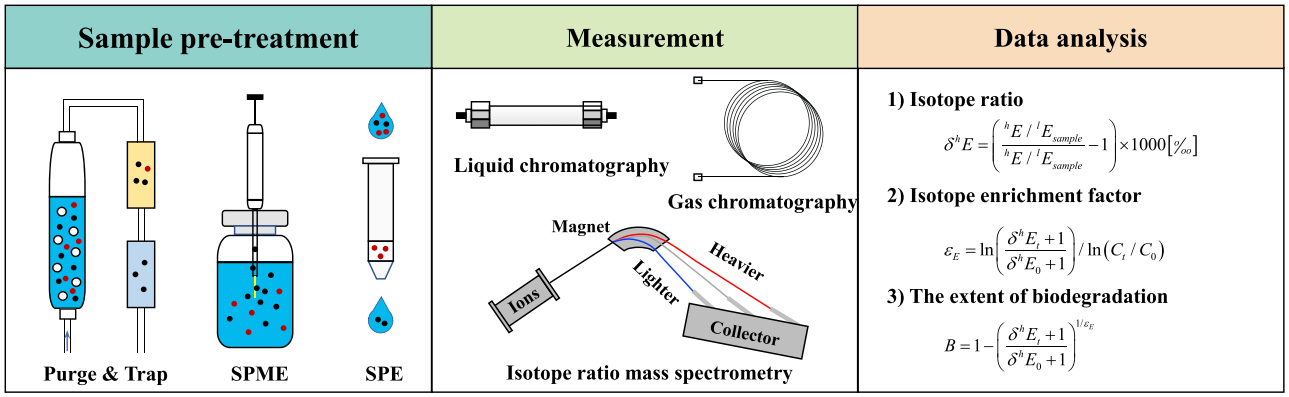

FIGURE 29.5 Schematic workflow of CSIA including sample pretreatment, instrumental measurement, and data analysis for characterization of isotopic composition of organic pollutants. The equations can be found in previous studies [84,88]. *CSIA*, Compound-specific isotope analysis.

were assessed by comparing the isotope ratio values of benzotriazoles ($\delta^{13}C$ and $\delta^{15}N$) and their derivatives of commercial standards with samples collected from tap water, wastewater treatment effluent, activated sludge, and dishwashing detergents [93]. Osenbrück et al. [94] applied major hydrochemistry, boron, $\delta^{18}O$, $\delta^{2}H$, and $\delta^{34}S$ of sulfate as the indicators for the origin of carbamazepine, galaxolide, and BPA in urban groundwater. They observed that the origin of those micropollutants was likely related to surface runoff infiltration, rather than the local river water infiltration to the Quaternary aquifer and sewer exfiltration.

Second, isotope fractionation can distinguish degradation pathways of micropollutants, such as abiotic versus biotic transformation and aerobic versus anaerobic degradation [88]. For example, in the study of Birkigt et al. [95], carbon isotope enrichment factors for SMX were $-0.6 \pm 0.1‰$ for biodegradation by *Microbacterium* sp. BR1 and $-2.0 \pm 0.1‰$ and $-3.0 \pm 0.2‰$ for direct photolysis at pH 7.4 and pH 5, respectively. Similarly, the degradation of methyl tert-butyl ether showed carbon isotope enrichment factors of around $-2‰$ and $-9‰$ for aerobic and anaerobic degradation, respectively, while hydrogen isotope enrichment factors clustered around $-40‰$ for aerobic and $-10‰$ for anaerobic degradation [96,97]. The differences in enrichment factors can be used to identify degradation pathways of those micropollutants in environmental samples.

Third, CISA can provide evidence for the occurrence and mechanisms of specific degradation reactions. Jin and Rolle [98] precisely captured dual element isotope trends of dichlorobenzamide, isoproturon (IPU), and diclofenac in different reaction pathways using the 2D-CSIA. For example, carbon and nitrogen isotope fractionation varied for IPU and its two TPs (isopropylphenyl isocyanate, ISO; dimethylamine, DMA) during microbial hydrolysis, resulting in different trends and slopes in a dual-isotope plot. In the plot, $\delta^{13}C$ values varied from $-33.3‰$ to $-29.8‰$ for ISO, $-29.7‰$ to $-18.34‰$ for DMA, and $-30.4‰$ to $-18.5‰$ for IPU; $\delta^{15}N$ ranged from 0.3‰ to 13.1‰ for ISO, $-5.0‰$ to 3.8‰ for DMA and $-7.4‰$ to $-2.6‰$ for IPU. In the study of Willach et al. [83], the combination of high-resolution Ms analysis with CSIA confirmed that SMX was hydroxylated at the anilinic nitrogen rather than at the aromatic ring during ozonation, as no carbon isotope fractionation was observed.

29.3.2 DNA-stable isotope probing technique

The radioactive and stable isotope tracers provide an understanding of degradation pathways and mechanisms of micropollutants. When significant microbial degradation is observed, the next challenge is to determine which individual members in the microbial community are active and how they interact. Combining stable isotope labeling methods with molecular biology approaches, SIP technology establishes connections between microbial communities and their metabolic functions toward target compounds by incubating environmental samples with the compounds labeled with heavy stable isotopes [99–101]. In this process, microorganisms in the samples utilize the labeled substrates, leading to the assimilation and synthesis of biomarkers (e.g., DNA, RNA, protein, and PLFA) containing heavy isotope labeling, among which DNA-SIP is the most widely applied. Following ultra-high-speed density gradient centrifugation, the high-density DNA components are separated from the naturally abundant lighter isotopes [102]. Through a series of analyses including extraction, isolation, and comparison [103], specific microbial taxa with transformation capacities of the target compound are identified within the samples (Fig. 29.6).

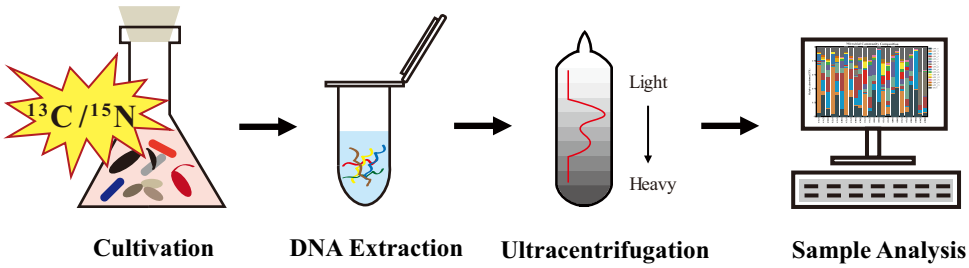

FIGURE 29.6 Schematic diagram of DNA-SIP workflow.

With the advancement of DNA-SIP and molecular biology techniques in studying the biodegradation processes of micropollutants, DNA-SIP has become a powerful tool for exploring degrading bacteria and their interaction mechanisms [99]. In this context, we have summarized studies conducted between 2018 and 2023 that employed SIP technology to identify degrading bacteria, elucidate degradation mechanisms, and explore metabolic pathways of micropollutants, including polycyclic aromatic hydrocarbons (PAHs), pesticides, antibiotics, and bisphenols (Table 29.1).

PAHs and their derivatives, due to their widespread contamination and ease of labeling, have served as indicator pollutants for identifying functional bacteria in specific environments at the early stage of SIP technology development [139]. In recent years, research using SIP technology for probing functional microorganisms involved in PAHs degradation mainly focused on naphthalene, phenanthrene, and pyrene, with phenanthrene being the most extensively studied. Li et al. [106–108,111,118] utilized SIP technology and molecular biology (e.g., high-throughput sequencing) to conduct a series of studies on phenanthrene-degrading microorganisms and their degradation mechanisms in complex environmental systems such as soil, exudates, and wastewater. In these studies, the genera *Pseudomonas, Sphingobium*, and *Rhodoplanes* were identified as phenanthrene-degrading bacterial taxa based on DNA profiling [106,107]. Additionally, a pure strain of *Acinetobacter tandoii* LJ-5 was isolated from wastewater, which did not directly participate in phenanthrene degradation, but enhanced its transformation by other microorganisms, providing a new perspective on the biological removal of phenanthrene [107]. Li et al. [118] utilized magnetic-nanoparticle-mediated isolation, combined with SIP and Raman-activated cell sorting, to significantly enrich active phenanthrene degraders, and in particular successfully isolate representative single cells.

Furthermore, SIP technology has led to the discovery of numerous functional microorganisms involved in the in situ degradation of other micropollutants such as pesticides, antibiotics, and bisphenols. These findings underscore the potential of cultivation-independent stable isotope-based molecular approaches in revealing the structure of degrading populations in complex microbial communities under natural conditions [140]. For instance, Lerner et al. [126] employed SIP technology to investigate in situ bacteria degrading linuron in soil. They demonstrated that linuron dissipation was significantly associated to *Variovorax* along with its linuron catabolic genes, and the process might involve synergistic cooperation between two *Variovorax* species. Apart from the reported *Variovorax*, *Ramlibacter* was also found for the first time as another genus of linuron-degrading bacteria [127]. Using DNA-SIP and protein-SIP, Ouyang et al. [134] confirmed incorporation of ^{13}C derived from ^{13}C-labeled SMX into proteins of bacteria from the families Intrasporangiaceae, Nocardioidaceae, and the order Solirubrobacterales, suggesting the crucial role of yet-uncultivated indigenous bacteria in the degradation of antibiotics.

29.3.3 Stable isotope probing-single-cell Raman spectroscopy technique

Although SIP operates independently of traditional separation and culture techniques, acquiring and further studying pure cultures of functional microorganisms are essential for the practical application of SIP research outcomes. SCRS provides biochemical fingerprints of bacteria at the single-cell level, capturing details such as nucleic acids, proteins, lipids, pigments, and polymers in a nondestructive manner [141]. When integrated with SIP techniques, stable isotopes from labeled substrates are incorporated into biomass and generated characteristic Raman shifts (Fig. 29.7), resulting from the substitution of light atoms with heavier stable isotopes in the chemical bonds of newly synthesized biomolecules [144,145]. The synergy of SIP with SCRS demonstrates remarkable potential in recognizing microbial single cells involved in pollutant transformation in complex environments [118]. To date, SIP-SCRS has been applied to investigate characteristics and functions of bacteria, as well as for differentiating and sorting cells based on isotopes of ^{13}C, ^{15}N, and ^{2}D [26,142].

TABLE 29.1 Application of ^{13}C-SIP technology in identifying degrading bacteria of micropollutants (2018–23).

Pollutants	Environmental matrix	Biomarker	Research methodology	Degrading bacteria
Polycyclic aromatic hydrocarbons (PAHs)				
Naphthalene [104]	Coal-tar contaminated groundwater	DNA	DNA-SIP, shotgun metagenomics, single-cell analyses	Six Naphthalene degraders
Naphthalene [105]	Crude oil-degrading enrichment	DNA	DNA-SIP, metagenomics, 16S rRNA gene sequencing	Unclassified Clostridiaceae species
Phenanthrene (PHE) [106]	PAH-contaminated wastewater	DNA	MMI-SIP, 16S rRNA gene sequencing	*Pseudomonas*, *Sphingobium*
PHE [107]	PAH-contaminated wastewater	DNA	DNA-SIP, 16S rRNA gene sequencing	*Rhodoplanes*, *Ammoniphilus*, *Sporosarcina*, *Hyphomicrobium*
PHE [108]	Ryegrass root exudates	DNA	DNA-SIP, 16S rRNA gene sequencing	Alphaproteobacteria, Nitrososphaera, Actinobacteria, *Sphingobacteriia*
PHE [109]	Various PAH-contaminated soils	DNA	DNA-SIP, 16 S rRNA gene sequencing	*Mycobacterium*
PHE [110]	Ryegrass-planted soil	DNA	DNA-SIP, metagenomics, 16S rRNA gene sequencing	Proteobacteria, Actinobacteria, Firmicutes
PHE [111]	Petroleum-contaminated soil	DNA	DNA-SIP, 16S rRNA gene sequencing	*Sphingomonas*, *Sphingobacterium*, *Acidovorax*, *Massilia*, *Flavobacterium*, *Cupriavidus*, *Aeromicrobium*, Chitinophagaceae
PHE [112]	PHE-contaminated soil	DNA	DNA-SIP, 16 S rRNA gene sequencing	*Acidovorax carolinensis* sp.
PHE [113]	PHE-contaminated sandy soils	DNA	DNA-SIP, 16S rRNA gene sequencing	Fungus: *Cryptococcus*, *Cladosporium*, Bacteria: *Tremellales*, *Marinobacter*, *Enterococcus*
PHE [114]	Petroleum-contaminated soil	DNA	DNA-SIP, 16S rRNA sequencing	*Xanthomonas*, *Williamsia*, *Gillisia*
PHE [115]	*Phragmites australis* rhizosphere	DNA	DNA-SIP, 16S rRNA gene sequencing	Rhizobiales, Rhodobacterales, Lactobacillales, Enterobacteriales
PHE [116]	Rhizosphere soil	DNA	DNA-SIP, metagenomics	22 PHE degraders
PHE [117]	Petroleum-contaminated soil	DNA	DNA-SIP, 16S rRNA gene sequencing	Find different PHE degraders
PHE [118]	Petroleum-contaminated industrial wastewater	DNA	MMI-SIP-RACS, metagenomics, 16S rRNA gene sequencing	Sterolibacteriaceae, Sphingomonadaceae

(*Continued*)

TABLE 29.1 (Continued)

Pollutants	Environmental matrix	Biomarker	Research methodology	Degrading bacteria
PHE [119]	Biochar	DNA	DNA-SIP, 16S rRNA gene sequencing	*Sphaerobacter*, Diplorickettsiaceae, *Pseudonocardia*, *Planctomyces*
PHE [120]	Biochar	RNA	RNA-SIP, 16S rRNA gene sequencing	*Methylobacterium*, *Xanthomonas*, *Kroppenstedtia*, *Scopulibacillus*, *Bautia*, *Lactobacillus*
Benz(a)anthracene [121]	PAH-contaminated oilfield soil	DNA	DNA-SIP, metagenomics	*Immundisolibacter*
Pyrene (PYR) [122]	PAH-contaminated oilfield soil	DNA	DNA-SIP, 16S rRNA gene sequencing	19 PYR degraders
PYR [123]	PYR-contaminated soils	RNA	RNA-SIP, 16S rRNA gene sequencing	13 well-known and two novel PYR degraders
Biphenyl [124]	Biphenyl-contaminated soil	Protein	Protein-SIP, single-cell analyses, multiomics	Uncultured Alphaproteobacteria clade UBA11222
PHE, Fluoranthene [125]	PAH-contaminated sediment	DNA	DNA-SIP, 16S rRNA gene sequencing	Six PAH degraders
Pesticides				
Linuron [126]	Agricultural soil	DNA	DNA-SIP, 16S rRNA gene sequencing	*Variovorax*
Linuron [127]	Pesticide-contaminated wastewater	DNA	DNA-SIP, 16S rRNA gene sequencing	*Ramlibacter*
Triclosan [128]	Anoxic/oxic system	DNA	DNA-SIP, 16S rRNA gene sequencing	*Methylobacillus*
Triclosan [129]	Activated sludge	DNA	DNA-SIP, metagenomics, 16S rRNA gene sequencing	*Sphingobium*
DDT [130]	Soils (presence of earthworms)	DNA	DNA-SIP, 16S rRNA gene sequencing	*Streptomyces*, *Streptacidiphilus*, *Dermacoccus*, *Brevibacterium*, *Bacillus*, *Virgibacillus*
Hexachlorocyclohexane (HCH) [131]	HCH-contaminated soil	Protein	Protein-SIP, shotgun proteomics	*Fusarium equiseti* K3, *Sphingobium* sp. S8
Thiabendazole (TBZ) [132]	TBZ-degrading bacterial consortium	DNA	DNA-SIP, metagenomics, 16S rRNA gene sequencing	*Sphingomonas*, *Hydrogenophaga*

(*Continued*)

TABLE 29.1 (Continued)

Pollutants	Environmental matrix	Biomarker	Research methodology	Degrading bacteria
Antibiotics				
SMX [133]	Wetland sediments	DNA	DNA-SIP, 16S rRNA gene sequencing	γ-Proteobacteria
SMX [134]	Pig farm-impacted soil	DNA	DNA-SIP, Protein-SIP, 16S rRNA gene sequencing	Intrasporangiaceae, Nocardioidaceae, Gaiellaceae, Solirubrobacterales
SMX [135]	Wetland sediments	DNA	DNA-SIP, Metagenomics	*Mycobacterium*, Burkholderiaceae, Rhodocyclaceae
Sulfonamide [136]	Antibiotic-contaminated wetland sediments	DNA	DNA-SIP, Metagenomics, 16S rRNA gene sequencing	*Bradyrhizobium*, *Gemmatimonas*, unclassified Burkholderiaceae
Bisphenols				
Bisphenol S [137]	Electrochemically active biofilms	DNA	DNA-SIP, Metabolomics	Electroactive microbes
BPA [138]	Effluent from wastewater treatment plant	DNA	DNA-SIP, 16S rRNA gene sequencing	*Variovorax* spp., *Pusillimonas*

BPA, Bisphenol A; *SMX*, sulfamethoxazole; *PAH*, polycyclic aromatic hydrocarbon.

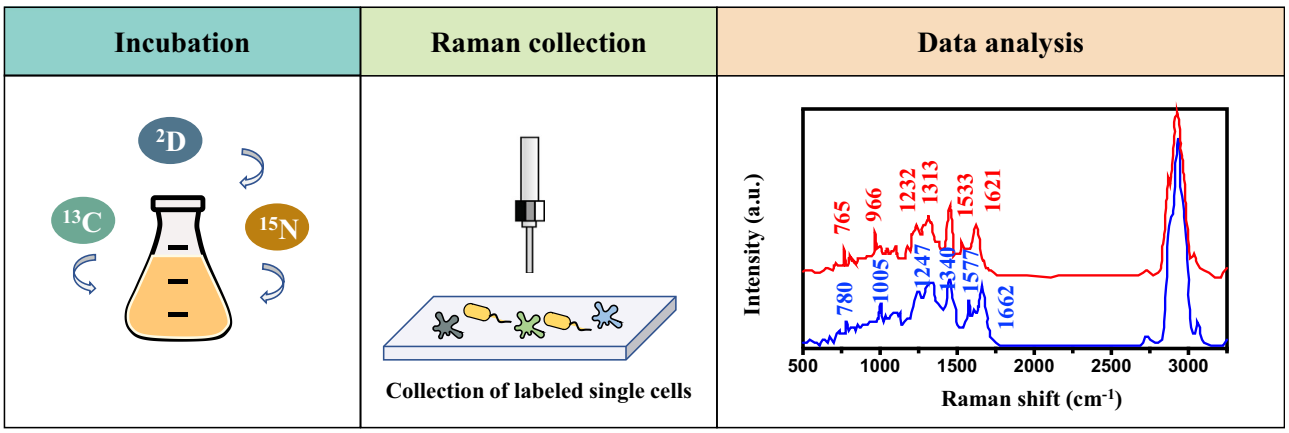

FIGURE 29.7 Schematic workflow of SIP-SCRS including incubation of samples with stable isotope-labeled compounds, collection of labeled single cells and data analysis [142]. The single-cell Raman spectra were collected from a previous study [143].

In SIP-SCRS, Raman spectra band shifts serve as indicators of isotope incorporation (Fig. 29.7). For example, surface-enhanced Raman scattering was for the first time combined with SIP in this environmental microbiology study to cultivate *E. coli* with ^{13}C-glucose, where a clear shift of band from 733 to 720 cm^{-1} in ^{13}C-*E. coli* cells was seen [146]. With respect to cell differentiation, SIP-SCRS has been applied to reveal nitrogen/phosphorus/carbon-fixing functional bacteria in complex soil and water communities [141,144,145], while limited attention was paid for micropollutant-resistant or degrading bacteria. Among the limited studies, SIP-SCRS was employed to significantly identify active phenanthrene degraders and successfully isolated the representative single cells (*Novosphingobium*) wastewater [118].

^{13}C-SIP-SCRS can be insensitive to detect cellular metabolic activity if the carbon incorporation is insufficient. The technique has a detection limit of about 10% $^{13}C/^{12}C + ^{13}C$ in a cell of interest [143]. In comparison, the application of heavy water (D_2O) to probe microbial community is sensitive enough to unravel general metabolic activity without cell reproduction [147]. D_2O-SIP-SCRS allowed the differentiation of antibiotic-resistant bacteria in environments containing antibiotics, as metabolically active bacteria can absorb heavy water, and thus generate a C−D band on the Raman spectra [142]. For example, antibiotic-resistant bacteria were identified in the River Thames within 24 hours by using D_2O-SIP-SCRS [148]. These results demonstrated that the percentage of bacteria resistant to carbenicillin, kanamycin, and both of the two antibiotics were 35 ± 5%, 28 ± 3%, and 25 ± 1% of the total bacterial population, respectively. In a recent study, D_2O-SIP-SCRS was applied along with advanced multivariate analysis and genotypic profiling to track in situ physiological evolution trajectory toward resistance [149]. The spectral changes of 1250 investigated cells were classified into four subsets: sensitive, intrinsic tolerant, evolved tolerant, and resistant, based on bacterial metabolic activity dynamics under ampicillin stress during evolution.

According to the fingerprint regions of SIP-SCRS, targeted bacteria can be sorted and collected for further genome amplification and sequenced purposes. To date, a variety of target bacteria have been successfully sorted from intricate environments, enabling subsequent species identification and functional gene analysis [142]. Three main types of SCRS technologies have been applied, including Raman-activated cell ejection, Raman tweezers, and microfluidic cell sorting [150]. The current omics technologies offer culture-independent methods that have been developed for phenotypic and genotypic analyses of microbial populations. Distinct from these technologies that generally characterize features of microbes as an overall community, SIP-SCRS can depict specific active species involved in micropollutant degradation [142]. By combining the strengths of SIP-SCRS with single-cell multiomics approaches a highly promising research avenue emerges. This synergy allows for complete genome of individual uncultivated species with specific function, eliminating the need for complex screening processes [142,151].

29.4 Conclusion

Unlike conventional analytical methods that rely on measuring parent compounds and their TPs, both radioactive and stable isotope tracers offer direct evidence of micropollutant degradation without the need to detect known or unknown TPs. Radioactive isotope tracers enable a comprehensive assessment of micropollutant fate by establishing a complete mass balance, even at environmentally relevant concentrations in complex matrices, including waters, soils, and sediments. Moreover, NER formation, an important fate of micropollutants and its TPs, can be quantitatively investigated. For instance, formation of micropollutant-derived NERs in soil has been demonstrated to be largely dependent on the soil property, redox conditions, microbial activity, and soil organisms.

Stable isotope fractionation can identify sources of micropollutants, distinguish degradation pathways of micropollutants, and unravel occurrence and mechanisms of specific degradation reactions, via CSIA, PSIA, and multielement CSIA. Furthermore, the synergy of SIP with SCRS has shown remarkable potential in identifying functional-yet-uncultivable single cells involved in pollutant transformation, understanding characteristics and functions of bacteria, and differentiating and sorting cells. For example, nitrogen/phosphorus/carbon-fixing functional bacteria and micropollutant-resistant or degrading bacteria have been identified or sorted in complex soil and water communities. In summary, the use of radioactive and stable isotope tracers can provide missing information on micropollutant degradation and cycling, and thus can drive the development of regulatory limits and risk assessment criteria for micropollutants in the environment.

While isotope tracers have significantly advanced our understanding of the fate of micropollutants, several limitations hinder their broader application: (1) The commercially available isotope-labeled compounds are costly and limited in variety. It is crucial to improve the synthesis efficiency and variety of isotope-labeled compounds. (2) Sample extraction and fractionation procedures are known to influence the true distribution of radioactive-labeled compounds in the matrices. Further developments in nuclear magnetic resonance spectroscopic techniques would facilitate NER studies by identifying the nature of binding sites and environments for sequestration in matrices [25]. (3) Application of CSIA to natural environmental systems remains an enormous challenge. From the analytical perspective, improvements in sample pretreatment and sensitivity as well as peak separation of GC−IRMS should be a focal point in future studies [152]. (4) Raman signal magnification is necessary to increase the throughput of SIP-SCRS for probing and sorting microbial consortia [142]. (5) Combination of SIP-SCRS with machine learning might enhance screening, classification, and visualization of uncultivated bacteria [143]. Addressing these challenges will significantly enhance the effectiveness and applicability of isotope tracers in elucidating micropollutant behavior in the environment.

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