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RESEARCH ARTICLE

Assessment of degradation mechanism of imidacloprid residues in grape rhizosphere soil by UHPLC-Orbitrap™-MS and its residual impact on soil enzyme activity

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Abstract

Imidacloprid (IM) is a systemic insecticide persistent in the environment and possesses a negative impact on the non-targeted ecosystem. The objective of the present study was to evaluate the dissipation and degradation mechanism of IM residues in grape rhizosphere soil and to investigate its residual effect on soil enzyme activity at different IM spiking levels. The half-life of IM residue in soil was 27, 36, and 43.5 days at a spiking level of 1, 10, and 50 mg kg^{-1}, respectively following a bi-phasic first + first-order dissipation kinetics. UHPLC-Orbitrap™-MS analysis by targeted metabolomics approach revealed that IM metabolites such as IM-amine analogue, guanidine (reduction), 5-hydroxy IM (hydroxylation), IM-Urea (oxidation), reduced NO analogue of IM (oxidation), and olefin of guanidine IM (dehydrogenation) were identified and proposed the degradation mechanism in grape rhizosphere soil. Toxicity of IM residues on five extracellular enzymes, viz., dehydrogenase, acid phosphatase, alkaline phosphatase, β-glucosidase, and urease revealed that activity of dehydrogenase, acid phosphatase, and alkaline phosphatase remained unaffected at 60th day of sampling. The β-glucosidase and urease were negatively affected throughout the incubation period indicating the influence of IM residues on carbon and nitrogen mineralization in soil. Thus, long-term exposure of IM to grape rhizosphere through soil drenching could affect soil enzyme activity which has a negative effect on the soil nutrient cycle and soil microbiome.

Keywords Imidacloprid · Dissipation · UHPLC-Orbitrap-MS · Grapevine · Non-targeted effect · Soil enzyme activity · IM metabolites

Abbreviations

IM	Imidacloprid
DHA	Dehydrogenase
HPLC	High-performance liquid chromatography
LC–MS/MS	Liquid chromatography-tandem mass spectrometry
UHPLC	Ultra-high-performance liquid chromatography

Introduction

Application of synthetic pesticides is integral part of commercial viticulture for the control of various pests and diseases. Imidacloprid (IM) (1-(6-chloro-3-pyridyl methyl)-N-nitroimidazolidin-2-ylideneamine), a neonicotinoid class of pesticide that acts as an insect neurotoxicant, has the potential activity against flea beetle, aphids, flies, thrips, white flies, etc. in various crops including grapes (Adak et al. 2012; Mahapatra et al. 2017a; Zamule et al. 2021). Due to its highly selective character and efficacy, it is more widely utilized in field crops to prevent the emergence of pests and insect resistance (Gao et al. 2021).

Pesticides applied are discharged into the environment where only a fraction of them reach the target pests, while the rest interfere with the environment and cause several non-target detrimental effects (Ramudu et al. 2011). Imidacloprid, a foliar and soil-drenching insecticide, is highly persistent and reported to have a half-life of 28 to 1250 days

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in soil having varying physicochemical properties (Goulson 2013). Interaction of pesticides with soil microbial community has an adverse effect on the production of the enzyme that plays a crucial role in decomposing organic matter and mineralization of the nutrients (García-Ruiz et al. 2008; Mishra and Behera 2008). Enzymes act as soil quality indicators which are used to measure the biological activity in response to pesticide contamination (Meena et al. 2020). The effect of pesticides on the rhizospheric microbiota can be determined with the study of enzymatic activity dynamics (Usharani and Lakshmanaperumalsamy 2016). The toxicity of pesticides on soil microorganisms is studied using enzymes, viz., dehydrogenase (DHA), acid and alkaline phosphatase, urease, and β-glucosidase which are responsible for biogeochemical cycles and nutrient mineralization. Dehydrogenase enzyme produced extracellularly in the soil is used to evaluate soil respiration and microbial biomass as well as for detecting changes brought on by alteration like the application of pesticides (Ataikiru et al. 2019). The conversion of phosphorus into inorganic form is brought by phosphatase present in the soil and has an influence on the rate of phosphorous cycle (Ataikiru et al. 2019). Ureases play a major role in the nitrogen cycle in soil by converting urea through hydrolysis into carbon dioxide and ammonia (Ataikiru et al. 2019). Another extracellular enzyme β-glucosidase is responsible for the carbon cycle by transforming complex organic matter into simple glucose monomers (Eivazi and Tabatabai 1988). Recent studies have shown that the application of imidacloprid changes soil microbial activity (Garg et al. 2021). Previous literature has reported that IM residues have declined the nitrification rate in soil (Deborah et al. 2013; Cycoń and Piotrowska-Seget 2015). Deborah and Madhuri (2013) reported that IM residues at higher doses reduce urease activity. Therefore, it is crucial to comprehend how pesticide residues in soil affect the enzymatic activity of soil. There is no such report available on the impact of soil-applied imidacloprid residues on enzymatic activity in grape rhizosphere soil which is important to understand the nutrient availability and mineralization in grape rhizosphere.

Impact of IM residue on soil microbial diversity and enzyme activity also depends on the degradation products generated in the process of dissipation. It is being reported that IM metabolites, viz., imidacloprid urea, 6-hydroxynicotinic acid, and 6-chloronicotinic acid persist in the environment for a longer period (Bacey 2000). Metabolism of imidacloprid was studied in sugarcane soil and reported to have toxic effects on non-target environments (Sharma and Singh 2013). IM degradation products in soil, viz., imidacloprid urea, 6-hydroxynicotinic acid, and 6-chloronicotinic acid, were persist in the environment for a longer period causing an adverse effect on soil microbiota and enzyme activity (Bacey 2000; Wang et al. 2014; Fernández-Gómez et al. 2011). According to published findings, IM and its degradation products impact the dynamics of soil microbiome and enzyme activities resulting in the reduction of soil biomass and soil enzymatic activities (Cycoń and Piotrowska-Seget 2015; Wang et al. 2014). Since the impact of IM residues on enzymatic activity also depends on degradation products generated in soil, its degradation mechanism also needs to be investigated in soil. To the best of our literature review, there are no published reports available on the degradation mechanism of imidacloprid in grape rhizosphere soil and its correlation to soil enzyme activity and nutrient mineralization.

Considering the above research gap, this study's objectives were to study the effect of imidacloprid residues and its metabolites on extracellular and intracellular enzyme activity in grape rhizosphere soil and a degradation pathway of IM in the soil is predicted with the help of degradation products identified through a targeted metabolomics approach by UHPLC-Orbitrap-MS analysis.

Materials and methods

Chemicals and instrument

The IM (98.55% purity) reference standard and its metabolite 6-chloro nicotinic acid (98.7%) were acquired from Dr. Ehrenstorfer GmBH, Augsburg, Germany. Solvents used, like ethyl acetate, methanol, toluene, and water, were of HPLC grade which were bought from Thomas Baker (Mumbai, India). The d-SPE cleanup sorbent Primary Secondary Amine (40-µm diameter, Bondesil) was supplied by Agilent Technologies, USA. Other reagents, viz. anhydrous magnesium sulfate (dried), calcium chloride ($CaCl_2$), sodium hydroxide (NaOH), and sodium chloride were procured from Merck India Ltd. (Mumbai, India). The water (resistivity > 18 M cm) used as the mobile phase was generated in-house (Sartorius, Gottingen, Germany). 0.2 µm PTFE filter (Thermo Fisher Scientific, Mumbai, India).

The equipment used for sample processing comprises a mixer grinder (Bajaj India Ltd., Mumbai), vortex mixer (Imperial Biomedicals, Mumbai, India), high-speed refrigerated centrifuge (Kubota Corp., Tokyo, Japan), micro-centrifuge (Kendro D-37520, Osterode, Germany), UHPLC-Orbitrap-MS (Thermo Fisher Scientific, Bremen, Germany), and Shimadzu 1700 UV–Vis spectrophotometer (Kyoto, Japan).

Collection of soil sample

The rhizosphere soil sample (free from IM and its metabolites) was collected from the 10–20-cm upper layer of a grapevine field located in the experimental organic vineyard

of ICAR-National Research Centre for Grapes, Pune, Maharashtra, India. The vineyard was located at latitude: 18.52°N, longitude: 73.86°E, elevation: 560 m MSL. During the soil collection, a maximum temperature of 28.2 °C, a minimum of 18.7 °C, and relative humidity of 72% were recorded. The soil was collected in a polythene bag and brought to the laboratory. Further soil was processed by air drying under shade and gentle grinding with hands to loosen the soil clumps which were passed through a sieve (2 mm) to remove the dried big roots and stones and to get uniform soil particles. Characteristics of the collected soil are as follows: clay texture, pH 8.05, organic carbon 1.30%, available nitrogen 78.4 mg kg^{-1}, phosphorus 42.1 mg kg^{-1}, potassium 2320.0 mg kg^{-1}, and sulfur 40.2 mg kg^{-1} (Tandon 2005).

In-vitro dissipation of imidacloprid in grape rhizosphere soil

In-vitro experiment

To study the dissipation pattern of IM, the methodology was followed as per previous literature with slight modification (Mahapatra et al. 2017a, Liu et al. 2006). A total of 500 g of soil were weighed in sterile polypropylene bottles. IM standard dissolved in methanol was fortified at a concentration of 1 mg kg^{-1} (resembles the concentration at the recommended dose of application), 10 mg kg^{-1} (resembles concentration considering the repeated application at higher doses), and 50 mg kg^{-1} (resembles the concentration due to accidental spillage) in soil and was mixed thoroughly to get sufficiently homogeneous distribution, and control was also maintained without pesticide (Sharma and Singh 2014; Cycon and Piotrowska-Seget 2015; Ge et al. 2017; Mahapatra et al. 2017a). Water was added daily to a certain amount to maintain the moist condition of the soil at field capacity level and bottles were stored at room temperature in dark conditions. All the treatments were replicated thrice. Five-gram soil each in triplicates was collected at regular intervals of 0 (2 h after spiking), 1, 3, 7, 10, 20, 30, 45, and 60 days and processed for IM residues and enzymatic assays (Mahapatra et al. 2017a; Mahapatra et al. 2017b).

Sample preparation for IM residue analysis

An in-house validated analytical method was followed for the IM extraction from the soil (Sabale et al. 2015). The soil sample (5.00 ± 0.10 g + 5 mL water) was weighed into a 50-mL centrifuge tube and to it, 10 mL acetonitrile was added and vortexed thoroughly for 2 min, then 4 g anhydrous magnesium sulfate and 1 g NaCl were added. The mixture was homogenized for 2 min on vortex and then centrifuged for 5 min at 5000 rpm. One milliliter of supernatant was cleaned with 25 mg PSA and 150 mg of $MgSO_4$. Tube was shaken vigorously for 30 s and centrifuged at 10,000 rpm for 5 min. The final vials were diluted filtered through a 0.2-µm PTFE filter and injected into an LC–MS/MS.

The soil samples were investigated with an LC–MS/MS system composed of Shimadzu UFLC coupled to API 4000 Qtrap MS/MS (Sciex, Foster City, CA, USA). A C-18 column (Luna -C18 (100 × 2.0 mm i.d., 3 µm)) was used for chromatographic separation. A mobile phase comprising of (A) water and (B) methanol, having 10 mM ammonium formate with 0.1% formic acid with a gradient program of 0–0.5 min 10% B phase, 0.5–2 min 10–70% B phase, 2–7 min 70% B phase, 7–7.5 min 70–10% B phase, and 7.5–10 min 10% B phase, was achieved. An injection volume of 10 µL with a flow rate of 400 µl min^{-1} and a column oven temperature of 40 °C was maintained.

Analytical method validation and quality control

A single laboratory method validation was executed for the residue investigation pertaining to specificity, limits of detection (LOD = signal-to-noise ratios of 3), the limit of quantification (LOQ = signal-to-noise ratios of 10), accuracy ($n = 6$), precision ($n = 6$), recovery (> 70%), and matrix effect (SANTE 2021). The matrix effect was determined by linking the analyte response in a standard solution to that of matrix extract spiked with the analyte at the same concentration. Accuracy and precision experiments were performed by fortifying the untreated soil sample in six replicates with imidacloprid at three concentration levels 0.01, 0.10, and 0.50 mg kg^{-1}. The repeatability precision was derived by figuring RSD (%) values associated with recovery.

Evaluation of dissipation kinetics

The residue data were subjected to linear and non-linear dissipation kinetics models using the subsequent mathematical equation by means of Table Curve 2D (v 5.01) program:

$$\text{First} - \text{order model} : [A]^t = [A]^1 \exp\left(-k^1 t\right)$$

$$\text{First} + \text{first} - \text{order model} : [A]_t = [A]^t \exp\left(-k_1 t\right) + [A]^2 \exp\left(-k_2 t\right)$$

where $[A]t$ is the concentration of analyte (mg kg^{-1}) at time t (days), $[A]^1$, $[A]^2$ = (0 days) degraded through first-order processes, and k_1 and k_2 are the degradation rate constants.

The half-life (DT_{50}) was determined using a first-order model, where DT_{50} (days) = ln (2) × k_1^{-1} (Thekkumpurath et al. 2020).

Residual effect of IM on soil enzyme activities

The effect of IM residue on soil enzymes, viz., dehydrogenase, acid and alkaline phosphatase, β-glucosidase, and

urease was analyzed in this experiment. Soil samples fortified with 1, 10, and 50 mg kg^{-1} with IM collected (5 g) on 0 (after 2 h of spike), 1, 3, 7, 10, 20, 30, 45, and 60 days were used to study enzyme activity. Dehydrogenase activity was determined using a method by Casida et al. (1964). Triphenyl tetrazolium chloride (TTC) is reduced to produce triphenyl formazan (TPF). The method is based on extraction with methanol and estimating TPF at 485 nm in a Shimadzu 1700 UV–Vis spectrophotometer. Determination of acid and alkaline phosphatase was performed using the methods of Tabatabai and Bremmer (1969) and Eivazi and Tabatabai (1977), respectively. Toluene (0.25 mL) and modified universal buffer (MUB) of pH 6.5 for acid phosphatase and pH 11 for alkaline phosphatase were added to the soil sample (1 g) and 0.05 M p-nitrophenyl phosphate (1 mL) and incubated for 1 h at 37 °C. Following the incubation period, 0.5 M CaCl2 and 0.5 M NaOH (4 mL) were added and centrifuged at 15000 rpm for 10 min. The resulting p-nitrophenol (PNP) was determined in a spectrophotometer at 400 nm. By employing the Eivazi and Tabatabai (1988) method, the substrate p-nitrophenyl-β-D-glucopyranoside was used to measure β-glucosidase activity. Soil samples (1 g) were mixed with MUB (4 mL) of pH 6 and toluene (0.25 mL) and incubated for 1 h at 37 °C. The subsequent steps were identical to those for the acid and alkaline phosphatase described above. Urease activity in soil was determined by the rate of urea hydrolyzing in soil (Rotini 1935). Soil (2.5 g) is placed in a test tube with the addition of toluene (0.1 mL), tris(hydroxymethyl)aminomethane (THAM) buffer, and 0.2 M urea (1 mL). Tubes were kept for incubation at 37 °C for 2 h. After the incubation period, KCL-Ag_2SO_4 solution (25 mL) is added and centrifuged at 5000 rpm for 5 min. The intensity of urea produced is estimated with a UV–Vis spectrophotometer. The listed enzymes were also examined in soils in triplicates that were not applied with IM as a treatment control.

Assessment of degradation mechanism of IM residues in soil

UHPLC-Orbitrap-MS analysis

An Orbitrap Q-Exactive mass spectrometer (MS) with an Ultimate 3000-series ultra-high-performance liquid chromatograph (UHPLC) connected was employed for identifying the targeted metabolites of IM. An Ascentis Express C18 (100 × 2.1 mm, 2.7 µm) column from Supelco was used for chromatographic separations. The mobile phase comprised of (A) water (100%) and (B) ACN (100%) acidified with 0.1% formic acid following a gradient program of the following: 0–1 min: 1% B, 1–10 min: 99% B, 10–11 min: 99% B, and 11.1–16 min: 1% B. The flow rate was maintained at 0.4 mL/min. Analysis was performed using a heated-electro-spray ionization (H-ESI) source in positive polarity having gas flow rate (sheath: 45; auxiliary: 8; sweep: 1); spray voltage: 3.50 kV; S-lens RF level: 50.0; capillary temperature: 320 °C; and heater temperature: 300 °C. The MS analysis was accomplished in full scan with a data-dependent MS/MS (ddMS2) acquisition at 17500 resolutions (m/z 200) operated at 18, 35, and 70 eV stepped collision energy. An automatic gain control (AGC) target of $1e^6$ was maintained. The reliability and reproducibility of each analysis were measured with the pooled quality control (QC) samples. The carryover and background noise were monitored with the blank samples (50% MeOH). Each sample (treated and control) was analyzed in three replicates.

IM metabolite target database

In soil, a high-resolution Orbitrap-LC/MS was utilized to assess the degradation mechanism through a targeted investigation with an in-house prepared database of the 23 reported metabolites of IM in literature established based on authentic standards as a comprehensive list (Thurman et al. 2013). The database consisted of molecular formula, exact mass $(M+H)^+$, with the data-dependent acquisition (MS/MS) mode. The database was further validated in-house using the certified reference standard of imidacloprid and its metabolite 6-chloronicotinic acid.

Data processing

The target analytes from the IM metabolites database were identified and confirmed based on accurate mass measurement of the precursor and characteristic fragment ions (threshold intensity > 5000, mass error (± 5 ppm), retention time (deviation ± 0.1 min), isotopic pattern match (> 90%), and molecular formula). The IM reference standards solutions were injected to check the retention time, MS spectra, and MS/MS fragments and validate the correct identification by the database prepared. The Tracefinder software (version 3.3, Thermo Fisher Scientific) was used to LC–MS data files with 3 replications. The automated data processing assisted in compound identifications with an established database of IM and its metabolites as per the confirmation criteria specified above. Furthermore, based on the metabolites identified, a degradation mechanism or degradation pathway for imidacloprid in grape rhizosphere soil is proposed.

Result and discussion

Analytical method validation

The residues were analyzed using method validation as per SANTE/11312/2021 guidelines (SANTE 2021). The

accuracy and precision (expressed as RSD) were evaluated in dried soil spiked with IM at three concentration levels, i.e., 0.01, 0.10, and 0.50 mg kg^{-1} in six replicates. The solvent standard and matrix match standard linearity was recognized in the range of 0.01–0.5 mg kg^{-1} with a correlation coefficient (R^2) of the calibration curve > 0.99. A LOD of 0.004 mg kg^{-1} and a LOQ of 0.01 mg kg^{-1} was established for IM residues in soil. The average recoveries (%) obtained were 85% (±4.53), 89% (±5.71), and 88% (±4.53) at 0.01, 0.10, and 0.50 mg kg^{-1} fortification levels, respectively. For quantification of any residue expected above the linearity limit, the samples were diluted appropriately and injected into LC–MS/MS and a proper dilution factor was applied while quantifying the residue. Matrix effect observed at 0.01 mg kg^{-1} was − 12.5% indicating suppression of the signal; hence, all the quantification of residues was performed by respective matrix match calibration.

Table 1 Evaluation of dissipation kinetics (1st + 1st and 1st order), dissipation constants, and half-life of imidacloprid in grape rhizosphere soil at different fortification levels

Kinetics model	Parameters	Imidacloprid		
		1 mg kg^{-1}	10 mg kg^{-1}	50 mg kg^{-1}
1st + 1st order	R^2	0.992	0.992	0.998
	a (mg kg^{-1})	− 2.650	4.387	19.347
	b (day^{-1})	0.377	1.318	2.420
	c (mg kg^{-1})	0.194	2.318	0.361
	d (day^{-1})	3.210	4.772	29.081
	DT_{50} (days)	27	36	43.5
1st order	R^2	0.968	0.960	0.996
	a (mg kg^{-1})	0.409	4.750	20.720
	b (day^{-1})	0.511	5.030	29.460
	DT_{50} (days)	21.5	53.0	44.5

In-vitro dissipation of imidacloprid in grape rhizosphere soil

The residue dissipation behaviors of imidacloprid pertaining to 1 mg kg^{-1}, 10 mg kg^{-1}, and 50 mg kg^{-1} are presented in Fig. 1 and Table 1 and summarized below. The initial residue of IM obtained was found to be 0.93, 10.48, and 50.86 mg kg^{-1} from treatments 1, 10, and 50 mg kg^{-1}, respectively. After 1 week, the IM was dissipated at 33.33%, 28.05%, and 18.54% from treatments 1, 10, and 50 mg/kg, respectively. IM residue was observed to dissipate at 48.38%, 47.80%, and 43.31% and further to 62.36%, 55.53%, and 55.24% from treatments 1, 10, and 50 mg/kg on the 30th and 60th days, respectively. The first and first + first-order kinetics equation was employed for IM residue dissipation kinetics. The results revealed that the IM residues followed first + first-order kinetics with R^2 values of 0.992–0.998 at varying concentrations. The half-life value evaluated by first + first-order kinetics of treatments 1, 10, and 50 mg kg^{-1} was 27, 36, and 43.5 days, respectively.

The persistence of pesticides depends on the type of soil, moisture, organic and inorganic content present, pH, and ground cover (Baig et al. 2012). The outcomes of the current investigation are in correlation with past findings that point to IM's destiny in soil. In the current experiment, IM followed a 1st + 1st-order dissipation with a faster initial phase. Furthermore, in the first 7 days, a faster degradation (33.33% dissipation) was observed at 1 mg kg^{-1} treatment compared to 28.05% at 10 mg kg^{-1} and 18.54% (slower degradation) at 50 mg kg^{-1}. However, this difference in the rate of dissipation became narrow at a later phase, i.e., at the end of the experiment (60 days), 62.36% dissipation was observed

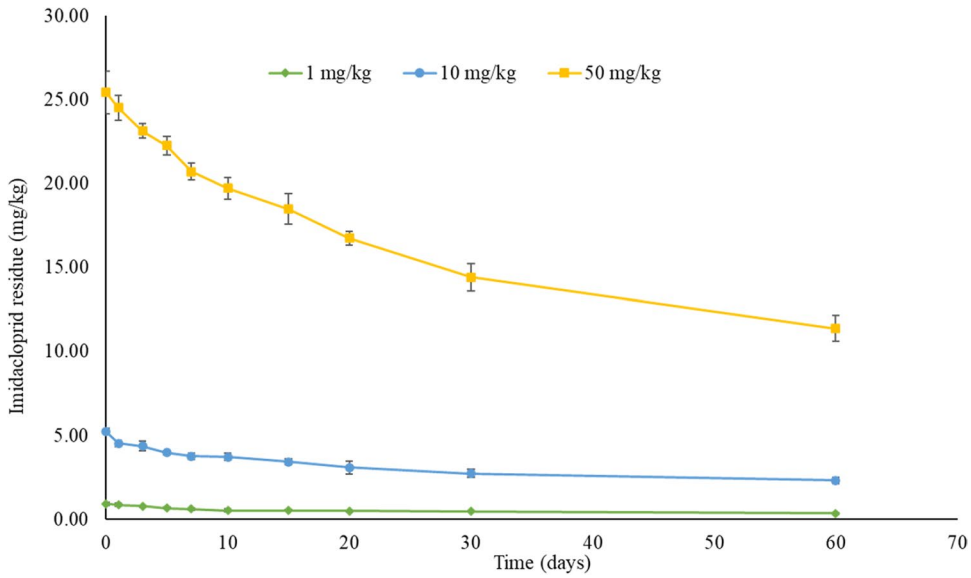

Fig. 1 In vitro dissipation of imidacloprid in rhizospheric soil of grapevine (error bars represent the standard deviation of three replications)

at 1 mg kg^{-1}, 55.53% at 10 mg kg^{-1}, and 55.24% observed at 50 mg kg^{-1}. This could be due to the fact that at higher concentrations, more IM is available for degradation as compared to 1 mg kg^{-1} due to IM adsorbed on clay minerals in the soil and released slowly which will be available for degradation and dissipation. The persistence of IM residue in clay soil could be explained by the pesticide's strong ability to bind to the clay particles. Texture of soil is an important factor that plays a role in the degradation of IM (Samnani et al. 2013). An earlier study of three types of soils, viz., alluvial soil, lateritic soil, and coastal alkaline soil showed a variation in the rate of persistence with a half-life ranging from 28 to 47.8 days suggesting increasing the soil's alkalinity makes the insecticide endure in the ground for longer (Sarkar et al. 2001). Present study soil sample shows high organic carbon (1.30%) which suggests the higher sorption of IM. Hence, the high organic carbon content and the alkaline pH of the soil could contribute to its high persistence in the studied soil (Sarkar et al. 2001; Liu et al. 2006; Sharma and Singh 2014). The past literature also suggests 1st + 1st-order bi-phasic dissipation for many pesticides in biological systems (Sable et al. 2015; Saha et al. 2016). The bi-phasic dissipation of IM in studied soil could be due to the partitioning of IM residues between soil solution and soil organic carbon or clay particles.

Impact of imidacloprid residues on soil enzyme activities

Impact of IM residues on dehydrogenase, acid phosphatase, alkaline phosphatase, urease, and β-glucosidase activity in soil integrated with three different concentrations of IM varied with time.

Effect on dehydrogenase activity

Dehydrogenase (DHA) is thought to reflect the metabolic activity of the soil and to be inversely correlated with the biomass of soil microbes. DHA which occurs intracellularly in live cells is thought to be the most helpful indication of soil microbial activity out of all the environmental enzymes. As DHA is not available freely in soil, except when cells die and are released into the environment, it may degrade more slowly than other extracellular enzymes under pesticide exposure. In this study, DHA activity was observed for a period of up to 60 days, and results show high activity of DHA initially between different concentrations of IM-spiked soil compared to the control (Fig. 2). Among the treatments, 50 mg kg^{-1} of IM in the soil had the strongest activity when compared with the control, further showed the highest activity on day 10 but subsequently dropped from the 20th day. In 10 mg kg^{-1} spiked soil, the enzyme activity exhibited a similar trend as in 50 mg kg^{-1} treatment. The lowest activity was observed at 1 mg kg^{-1} when compared to other treatments and control. After day 20, the enzyme activity was decreased showing a significant difference with respect to the control. High activity of DHA on the 20th day of sampling in different doses of IM suggests that IM may be used as a potential food source by soil microbes and increase its cell division. This could be due to the fact that DHA is an intracellular enzyme, which will come in contact with pesticide only after the lysis of bacterial/microbes cell walls under the influence of pesticide application. However, this effect was not sustainable as DHA activity was found to decrease after 20 days of incubation. This indicates there is a negative consequence of IM on soil microbe activity after 20 days. Also, the impact of pesticides varies depending on the soil's microbial diversity and type. Previous findings have reported IM has a negative effect on dehydrogenase (Cycoń and Piotrowska-Seget 2015; Wang et al. 2014). Low dehydrogenase levels in soil treated with IM may be linked to the loss of the insecticide-sensitive microbial population. However, the impact was non-significant on the 60th day of sampling. Furthermore, as they degrade, the dehydrogenases released by diseased cells do not build up in the soil (Cycoń and Piotrowska-Seget 2015).

Effect on acid and alkaline phosphatase activity

Effect on acid phosphatase activity remained unaffected in treatments when compared with control (Fig. 2). Temporary enhancement in the activity was observed which gradually decreased with time and at the end of the experiment, there was no significant difference with respect to control. Initially, activity in 1 mg kg^{-1} and 10 mg kg^{-1} concentrations was more prominent than in 50 mg kg^{-1} concentration, which shows residues present in the soil are utilized as a carbon source by soil micro-organisms. The effect on alkaline phosphatase activity shows negligible difference in all three concentrations when compared with control. In the studied soil, activity up to day 5 was observed to have similar activity as control but on day 10, enzyme activity in 50 mg kg^{-1} was inhibited while the other two concentrations remain unaffected with respect to control. On day 20, the activity was regained, and on the 60th day of the experiment, all treatments showed the same alkaline phosphatase activities as the control. Reports show phosphatase is active in clay type of soil which protects against denaturation. The conclusion is no discernible change in daily activities was seen in any of the examined soils; this was most likely caused by the soil's high colloidal content and buffering ability. Acid phosphatase is an extracellular enzyme that, due to fast breakdown, is only present in the soil for a brief period (Sarkar et al. 1989). Despite this, certain free enzymes may be sustained by adhering to soil mineral particles or absorbed into humic substances (Burns 1986). Earlier reports predict

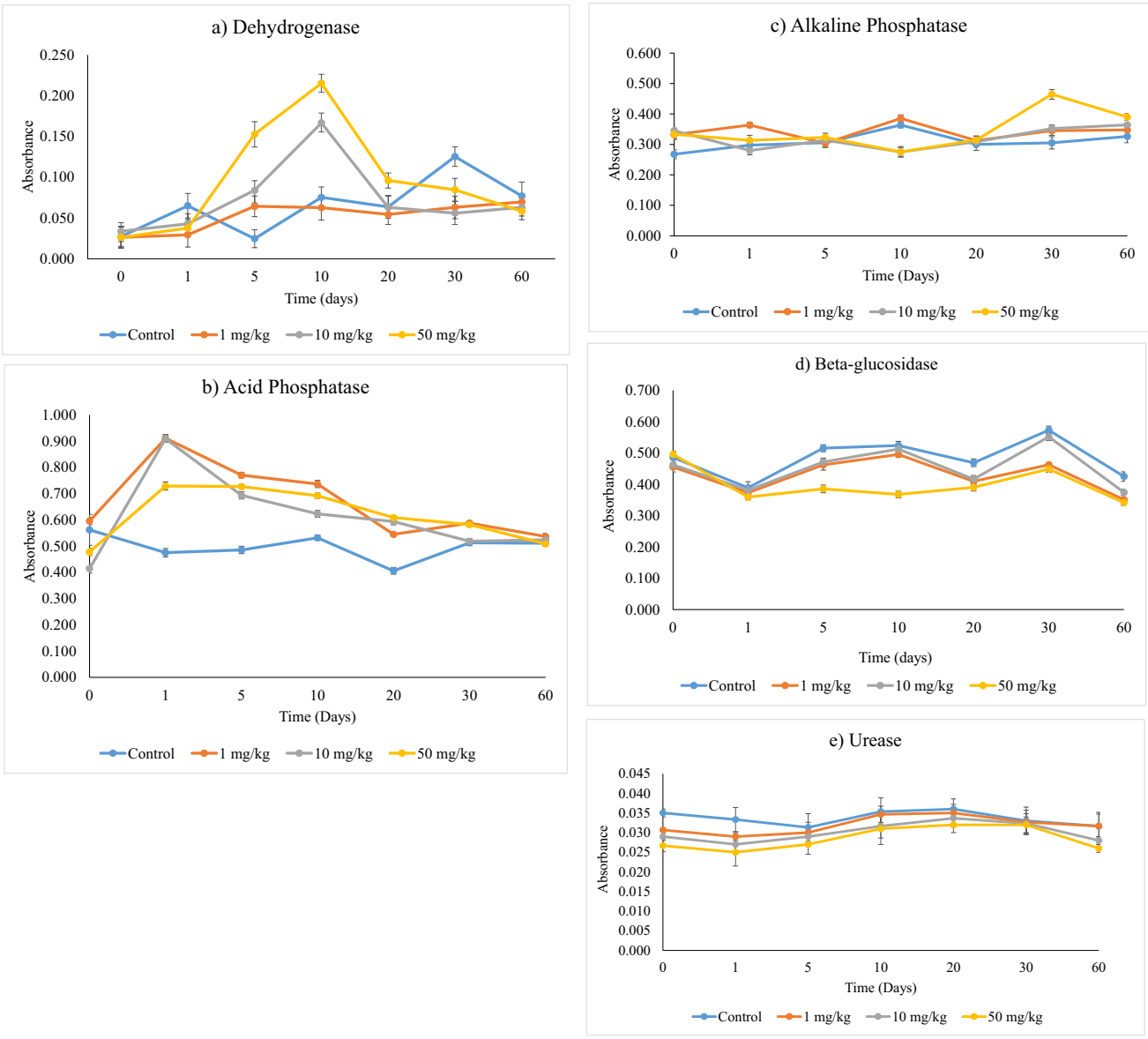

Fig. 2 Effect on extracellular enzymes in grapevine soil under different dosages of imidacloprid (error bars represent the standard deviation of three replications), were, **a** dehydrogenase, **b** acid phosphatase, **c** alkaline phosphatase, **d** beta-glucosidase, and **e** urease

that minerals and humic substances in the soil might be the reason for a decline in enzyme activity. Acid phosphatase activity varies as it depends on pH and type of soil. The soil under study has a high level of organic matter and a higher pH; thus, when the experiment first commenced, an increase in activity indicated that the environment was suitable for sustaining which gradually decreased further and became non-significant on the 60th day.

Effect on β-glucosidase activity

Activity of β-glucosidase was significantly affected by IM applied in three different concentrations over a period of time (Fig. 2). Initially, on 0 day, the activity was observed to be affected non-significantly in all the treatments. Later up to 20 days, the activity was found to be significantly inhibited compared to the control and the effect was more pronounced at higher fortification levels. Furthermore, until the completion of the experiment (60 days), the inhibitory effect was sustained compared to the control with more pronounced inhibition at 50 mg kg^{-1} fortification. This increase in the inhibition activity of β-glucosidase could be due to the adsorption of IM on the organic material of the soil and further inhibit β-glucosidase as it is an extracellular enzyme. Mahapatra et al. (2017a, b) observed similar results,

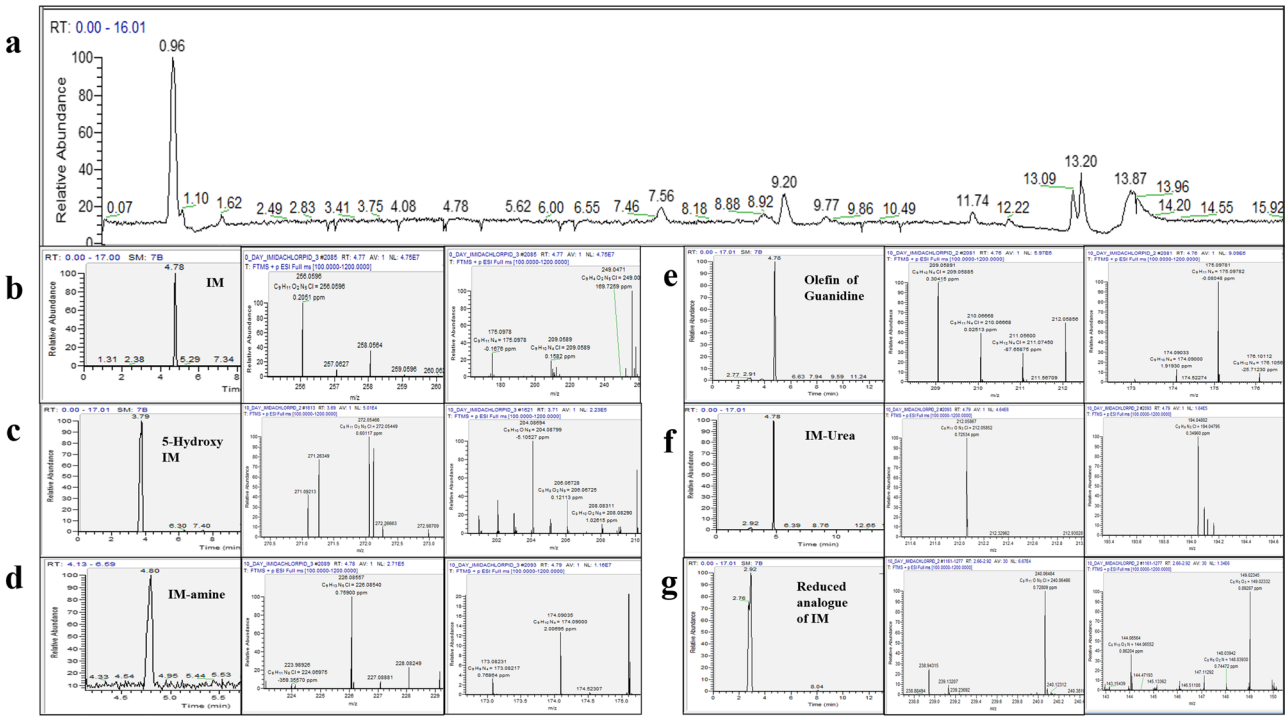

Fig. 3 Identification of imidacloprid metabolites in grape rhizosphere soil by UHPLC-Orbitrap-MS analysis by extracted ion chromatogram (XIC), isotopic mass spectra and MS^2 mass spectra of imidacloprid and its identified metabolites; **a** Total ion chromatogram of acetonitrile extract of soil spiked with imidacloprid at 10 mg kg^{-1}; **b**-**g** imidacloprid and its identified metabolites

where β-glucosidase activity reduced over time with the application of IM. β-glucosidase enzyme is associated with the biogeochemical cycle of carbon which acts on organic matter in soil and converts complex cellobiose to simple glucose (Riah et al. 2014). This end product is utilized by soil microorganisms as a carbon source. Therefore, a decline in the production of β-glucosidase activity may suggest that soil microbe involved in carbon mineralization may be affected by IM residues resulting in a reduction in available carbon in soil.

Effect on urease activity

Urease activity was significantly affected in all three treatments of IM whereas control showed high activity (Fig. 2). IM-spiked in studied soil observed inhibition in

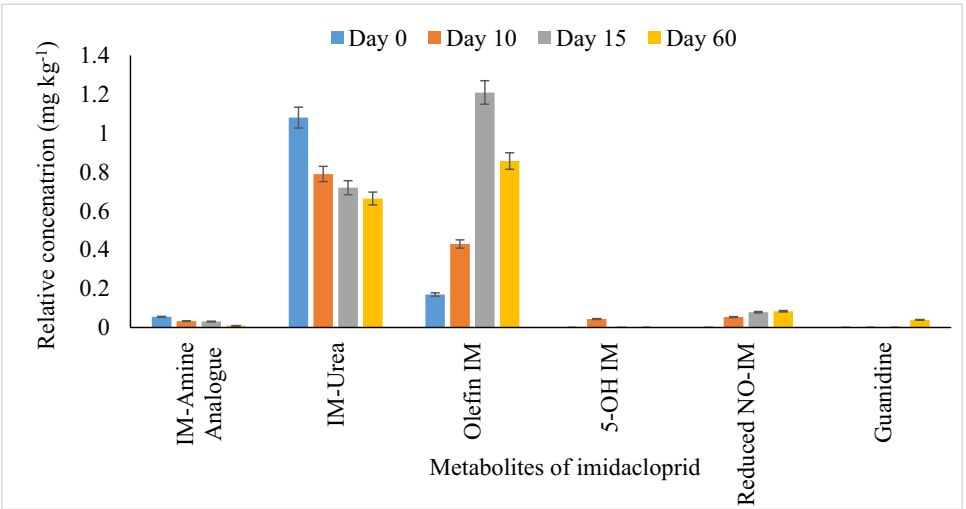

Fig. 4 The relative abundance of imidacloprid metabolites in grape rhizosphere soil at different time intervals (error bars represent the standard deviation of three replications)

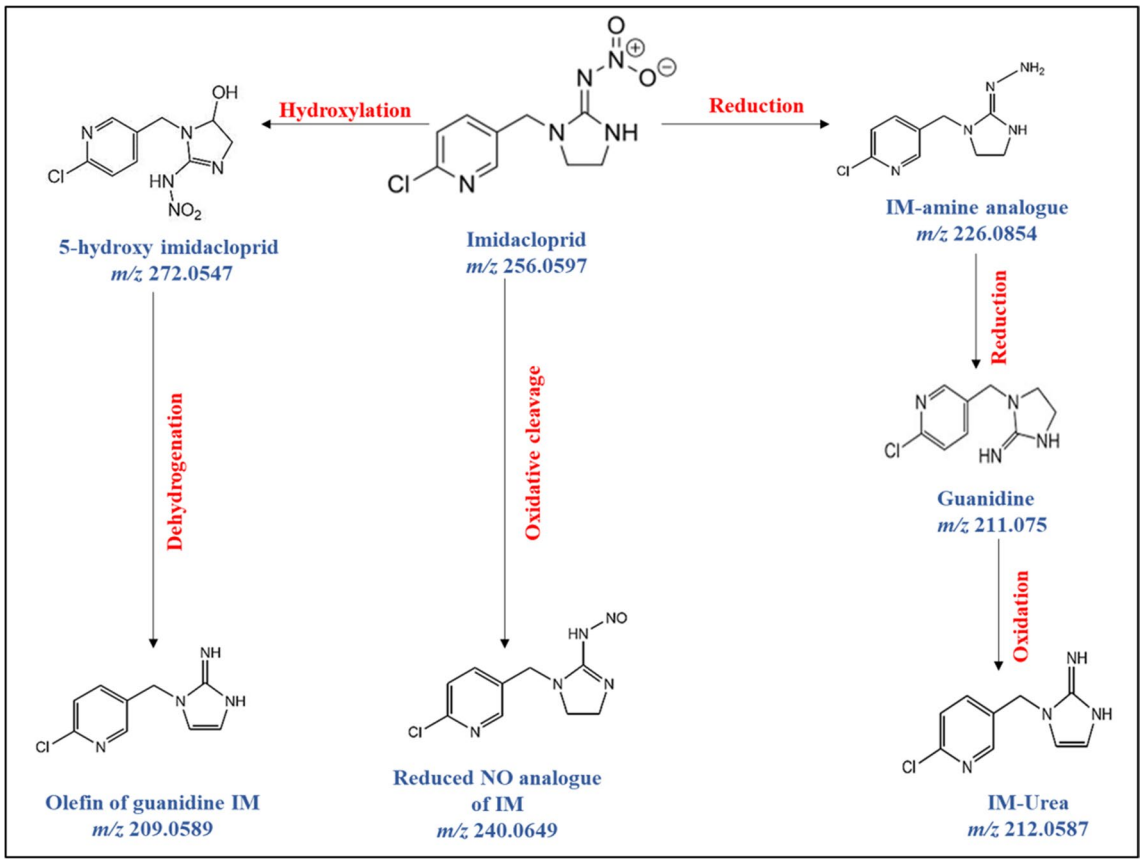

Fig. 5 Proposed degradation pathway of imidacloprid residues in grape rhizosphere soil

urease activity throughout the experimental period and the inhibition effect is more pronounced in 50 mg kg^{-1} when compared with the control soil indicating its concentration dependency. The inhibition observed remains almost static throughout the experiment indicating the long-term impact of IM residues on urease activity. This extracellular enzyme binds to available inorganic and organic soil colloids making them persist in the environment. Urease activity was most affected among all the enzyme activities studied in the experiment. It can be assumed that a component of these enzymes may be locked up in proliferating bacteria, non-proliferating microbes, in connection with/or in cell debris. Mahapatra et al. (2017a, b) reported urease activity was affected initially in different treatments of IM but the activity seems to be stabilized till the incubation period. However, in our study, the inhibition of urease activity was prolonged until 60 days of sampling. Urease is an extracellular enzyme that is involved in the nitrogen cycle. This enzyme hydrolyses urea present in soil into carbon dioxide and ammonia (Riah et al. 2014). These key components regulate the nitrogen supply to plants. Application of IM inhibits urease activity and reduces urea hydrolysis in soil resulting in reduction of available nitrogen in grape rhizosphere. Hence, application of IM in grape rhizosphere through drenching may affect the available carbon and nitrogen in soil indicating an over-fertilizer application to meet the crop requirement. However, this needs to be further evaluated through field experimentation.

Evaluation of imidacloprid metabolites at different time intervals

A compound library for IM and its potential degradation product was prepared according to Thurman et al. (2013) (Supplementary Table 1). LC-HRMS analysis was carried out to find the potential degradation pathway of IM (Fig. 3 and Fig. 4). The results revealed the parent compound IM (*m/z* 256.0597) and its metabolites, namely IM-amine analogue (*m/z* 226.0854), IM-Urea (*m/z* 212.0587), olefin of guanidine IM (*m/z* 209.0589), 5-hydroxyimidacloprid (*m/z* 272.0547), reduced NO analogue of IM (*m/z* 240.0649), and guanidine (*m/z* 211.075). The relative concentration of each metabolite obtained in HRMS analysis revealed the metabolite abundance at different days (days 0, 10, 15, 60) varied with parent ion concentration (Fig. 4). Among

them, imidacloprid urea concentration reduced gradually from 0 to 60 days with 1.081 mg kg^{-1} on day 0 to 0.66 mg kg^{-1} on day 60. On days 0, 10, and 15, IM-amine were observed. IM-olefin concentration was high, i.e., 1.52 mg kg^{-1} at day 15. Reduced NO analogue of IM was increased from days 10 to 60. The metabolites observed at different time points suggested the enzymatic pathway alteration by microbes or physicochemical properties of the soil.

Imidacloprid degradation mechanism in grape rhizosphere soil

Based on the metabolites obtained during targeted analysis, established degradation pathway of IM in grape rhizosphere soil (Fig. 5). Generally, imidacloprid degradation pathways vary significantly besides some of the metabolites are more toxic and persistent than the parent pesticide. In our study, nitro-group reduction of IM showed the nitrosamine derivative, i.e., IM-amine analogue. Then it was reduced to desnitro imidacloprid (guanidine), which was further oxidized to IM-urea which was found during 0 to 60 days with decreasing concentration, where urease enzyme activity was high. While IM was converted to 5-hydroxy IM which was prevalent at day 10, by hydroxylation and then its dehydrogenation formed the olefin of guanidine IM, found at days 0, 15, and 60. The next phase of IM degradation was found in this experiment where IM was degraded to produce a reduced NO analogue of IM by oxidative cleavage, during days 10, 15, and 60. Previous reports suggested the degradation of IM through microbes majorly depends on two metabolic pathways, viz., oxidation and nitro-reduction (Pang et al. 2020). In our study, we found both pathways that suggested the IM degradation pathway in the rhizosphere. The metabolite of IM produced as desnitro/guanidine intermediates is ten times more toxic than IM but subsequent conversion of these metabolites into IM-urea is nontoxic (Pandey et al. 2009; Phugare et al. 2013; Sharma and Singh 2014). In the presence of sucrose, the bacterial isolate *Stenotrophomonas* converted IM to the olefin metabolite through hydroxylation and dehydrogenation (Dai et al. 2010). Olefin, 4-hydroxy imidacloprid, and 5-hydroxy IM are the imidacloprid metabolites that have been recorded the most frequently (Hussain et al. 2016). In our study, we have reported olefin and 5-hydroxy IM also. Hence, overall, the study gives insights into the imidacloprid degradation pathway by its possible degradation of various metabolites in rhizosphere soil.

Conclusion

The persistent behavior of imidacloprid in grape rhizospheric soil and its effect on soil enzymatic activity supports the evidence of the pesticide's lethal effect on non-target organisms. In this investigation, the longer persistence of imidacloprid with respect to different concentrations was observed with a half-life of 27–53.5 days. Furthermore, the degradation mechanism of imidacloprid in the grape rhizosphere was established through the identification of IM-metabolites, viz., IM-amine analogue, IM-urea, olefin of guanidine IM, 5-hydroxy imidacloprid, reduced NO analogue of IM, and guanidine by a targeted metabolomics approach. The residual effect of imidacloprid showed a negative effect on β-glucosidase and urease extracellular enzymes, while the impact on dehydrogenase, acid- and alkaline phosphatase remained non-significant at the end of the experiment even though a temporary enhancement was observed in the initial phase. Inhibition of β-glucosidase and urease activity involved in the carbon and nitrogen cycle in the soil might affect the C:N ratio in the soil; hence, the availability of crucial nutrient uptake gets impacted. This may also lead to the accumulation of non-available organic carbon and nitrogen in the soil affecting the nutrient imbalance. Hence, a long-term drenching application of imidacloprid may impact the availability of nitrogen and organic carbon in viticulture which needs to be further validated through field experiments.

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Author contribution

- Conceptualization, methodology, design, and final manuscript: Ahammed Shabeer Thekkumpurath;
- Execution of experiment, statistical evaluation, and preliminary draft preparation: Prabhavati Ghotgalkar, Sachin Ekatpure, Anita Pardeshi, Vrushali Bhanbhane, and Pushpa Deore

Data availability Data associated with the manuscript will be available on request.

Declarations

The work does not include any human participants and/or animals.

Ethical statements The authors confirm that:

- The manuscript has not submitted to more than one journal (*Journal of Environmental Science and Pollution research*) for simultaneous consideration.

• The submitted work is original and not has been published elsewhere in any form or language (partially or in full).
• Results are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.
• No data, text, or theories by others are presented as if they were the author's own ("plagiarism"). Proper acknowledgements to other works are given.

Consent to participate Not applicable.

Consent to publish Not applicable.

Competing interests The authors declare no competing interests.

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