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Simultaneous removal of neonicotinoid insecticides by a microbial degrading consortium: Detoxification at reactor scale

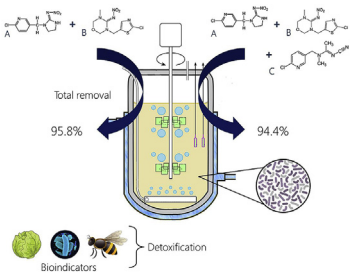
Gabriel Rodríguez-Castillo [a], Marvin Molina-Rodríguez [a], Juan Carlos Cambronero-Heinrichs [a], José Pablo Quirós-Fournier [b], Verónica Lizano-Fallas [a], César Jiménez-Rojas [a], Mario Masís-Mora [a], Víctor Castro-Gutiérrez [a], Iray Mata-Araya [b], Carlos E. Rodríguez-Rodríguez [a, *]

[a] Centro de Investigación en Contaminación Ambiental (CICA), Universidad de Costa Rica, 2060, San José, Costa Rica
[b] Centro Nacional de Innovaciones Biotecnológicas (CENIBiot), CeNAT-CONARE, 1174-1200, San José, Costa Rica

HIGHLIGHTS

- Microbial consortia capable to degrade imidacloprid were isolated.
- Cross degradation of other neonicotinoid insecticides was achieved.
- Reactor scale treatment (STBR) removed binary and ternary mixtures of neonicotinoids.
- The treatment partially detoxified the polluted matrix towards honeybees.
- Partial detoxification was achieved on seed germination and *Daphnia magna* tests.

GRAPHICAL ABSTRACT



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ABSTRACT

Neonicotinoid insecticides show high persistence in the environment, and standard biological approaches such as biopurification systems have shown mostly inefficient removal of such compounds. In this work, soil pre-exposed to imidacloprid was used to obtain presumptive imidacloprid-degrading consortia. Cometabolic enrichment yielded a microbial consortium composed of eight bacterial and one yeast strains, capable of degrading not only this compound, but also thiamethoxam and acetamiprid, as demonstrated in cross-degradation assays. The biological removal process was scaled-up to batch stirred tank bioreactors (STBR); this configuration was able to simultaneously remove mixtures of imidacloprid + thiamethoxam or imidacloprid + thiamethoxam + acetamiprid, reaching elimination of 95.8% and 94.4% of total neonicotinoids, respectively. Removal rates in the bioreactors followed the pattern imidacloprid > acetamiprid > thiamethoxam, including >99% elimination of imidacloprid in 6 d and 17 d (binary and ternary mixtures, respectively). A comprehensive evaluation of the detoxification in the STBR was performed using different biomarkers: seed germination (*Lactuca sativa*), bioluminescence inhibition (*Vibrio fischeri*), and acute oral tests in honeybees. Overall, ecotoxicological tests revealed partial detoxification of the matrix, with clearer detoxification patterns in the binary mixture. This biological approach represents a promising option for the removal of neonicotinoids from agricultural wastewater; however, optimization of the process should be performed before application in farms.

* Corresponding author.
E-mail address: carlos.rodriguezrodriguez@ucr.ac.cr (C.E. Rodríguez-Rodríguez).

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1. Introduction

The inappropriate application of pesticides and the undesirable management of their residues can result in contamination of soil, surface and groundwater (Kibblewhite et al., 2008; Kreuger and Törnqvist, 1998; Wang et al., 2019). On-farm pesticide handling is particularly risky as a point source of contamination of water compartments (Castillo et al., 2008), related to inadequate preparation of pesticide field-working solutions, filling and cleaning of spraying equipment, and management of pesticide residues after application on crop fields (Chen et al., 2014).

Among the pesticides of worldwide use, neonicotinoids represent a current environmental concern. They comprise a broad chemical group of insecticides that have been widely used in agricultural, commercial, residential, and veterinary practices to control pests in crops such as grains, cereals, beets and potatoes (Goulson, 2013). Their mode of action depends on the activation of post-synaptic nicotinic acetylcholine receptors (nAChR) in target insects or some non-target organisms (Casida and Durkin, 2013; Chen et al., 2014). Nonetheless, most of these compounds are persistent in the environment, as they exhibit long half-lives in soil, which vary widely among the chemical compound, soil type, and across studies; i.e. 31–450 d for acetamiprid (European Commission, 2004); 28–1250 d for imidacloprid (Sarkar et al., 2001); and 7–353 d for thiamethoxam (Robinson, 2001).

Approximately 80–98% of the amount of applied neonicotinoids is not absorbed by the crop, and consequently these compounds may reach the soil; furthermore, significant amounts of pesticide-containing particles are released by drilling machines during sowing (Tapparo et al., 2012). Such events result in the exposure of non-target organisms to sublethal doses of pesticides. Of particular concern is the negative effect exerted by neonicotinoid residues on honey bees, causing the decline in their populations (Christen et al., 2016; Fairbrother et al., 2014; Krupke et al., 2012; Yang et al., 2008; Zhu et al., 2019). The critical affectation of pollinators has led to an European Union decree that banned, since the end of 2018, the use of clothianidin, thiamethoxam and imidacloprid on crops pollinated by bees (Declan, 2018).

Different methods have been developed to remove pesticides from wastewater and consequently prevent pesticide contamination of environmental compartments. Physicochemical processes have demonstrated efficient removal of neonicotinoids, including heterogeneous and homogeneous photocatalysis (Kitsiou et al., 2009; Mahmoodi et al., 2007), photo-Fenton (Malato et al., 2001) and hydrodynamic cavitation reactors (Patil et al., 2014). However, there are few reports focused on the biological degradation of these pesticides (Hussain et al., 2016).

The use of biopurification systems has been regarded as an efficient approach for pesticide removal from agricultural wastewaters; however, extremely slow or negligible elimination has been usually reported for neonicotinoids in these systems (Huete-Soto et al., 2017; Masís-Mora et al., 2019; Rodríguez-Castillo et al., 2018). For this reason, the search for new biological strategies to achieve the swift removal of neonicotinoid insecticides is a matter of current concern. It is known that microbial consortia represent promising candidates for the removal of organopollutants; moreover, microbial degradation of pesticides is sometimes only effective when microorganisms are grouped in consortia and not as individual isolates (Akbar et al., 2014; Castro-Gutiérrez et al., 2016; Wolfaardt et al., 2007). In the particular case of neonicotinoids, Sharma et al. (2014) used a mix of bacterial isolates (*Bacillus aerophilus* and *Bacillus alkalinitrilicus*) for the lab-scale bioaugmentation of soil to degrade imidacloprid, resulting in half-lives ranging from 13 to 16 d.

Even though the removal of parent compounds is pursued in a degradation process, the production of toxic transformation products (of similar or higher toxicity than the original compound) represents a potential risk (Farré et al., 2008). Therefore, the ecotoxicological monitoring during a bioremediation process comprises a global, though not frequently applied approach to determine the detoxification of contaminated matrices (Gikas et al., 2018; Rodríguez-Rodríguez et al., 2017).

The aims of this study included obtaining an imidacloprid degrading consortium from pre-exposed agricultural fields and assessing its capacity to cross-degrade other neonicotinoid insecticides (thiamethoxam and acetamiprid). The performance of the microbial consortium was assayed at reactor scale using mixtures of insecticides; to estimate the potential detoxification due to pesticide elimination in the system, ecotoxicological assays (including tests on honeybees, seed germination and bioluminescent bacteria) were performed. Data here presented provide important knowledge on the design of biological approaches to promote the elimination of highly toxic and persistent pesticides such as neonicotinoids.

2. Materials and methods

2.1. Chemicals and reagents

Analytical standards imidacloprid ((*E*)-1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine) (99.5% purity), thiamethoxam ((*EZ*)-3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine) (99.5%), acetamiprid ((*E*)-*N*1-((6-chloro 3-pyridil)methyl)-*N*2-cyano-*N*1-methylacetamidine) (99.5%), and 6-chloronicotinic acid (6-chloropyridine-3-carboxylic acid) (99.2%) were obtained from Chem Service Inc. (West Chester, Pennsylvania, USA). Commercial imidacloprid (Manager®, 35% w/v), thiamethoxam (Engeo®, 24.7% w/v) and acetamiprid (Acetamiprid 20 SP®, 20% w/w) were purchased from a local market. Acetonitrile and methanol of HPLC grade, and formic acid (purity 98–100%) were obtained from Merck (Darmstadt, Germany).

2.2. Experimental procedures

2.2.1. Isolation of imidacloprid degrading microbial consortia and cross-removal of thiamethoxam and acetamiprid

A selective-enrichment procedure (Abraham and Silambarasan, 2013) was performed to obtain imidacloprid-degrading microbial consortia from soil pre-exposed to this insecticide. Soil samples were collected from the upper soil layer (0–20 cm) in two watermelon fields with a history of imidacloprid application, in San Mateo, Alajuela, Costa Rica. Six grams of soil were inoculated in three flasks containing 300 mL of either sterile Bushnell-Haas (BH) broth (pH = 6.5) without C-source (BH-C) (Bushnell and Haas, 1941), BH with added glucose (2 g L^{-1}) without N-source (BH-N), or BH without N- or C-source (BH-CN). Each system was supplemented with imidacloprid (50 mg kg^{-1}) and incubated in orbital shaking (28 °C, 160 rpm) for one week in the dark (first passage of the enrichment culture, P1). After incubation, 3 mL of this suspension were transferred to 300 mL of fresh medium and incubated as previously described for one week (P2). Third and fourth weekly passages (P3 and P4) were carried out similarly to obtain three degrading consortia per soil sample (C1, C2; N1, N2; CN1, CN2). Individual strains that make up the relevant imidacloprid-degrading microbial community were isolated by inoculating serial dilutions of the P4 suspension on replicate Trypticase Soy Agar plates (TSA, DIFCO Laboratories, Detroit, MI) and the respective

type of BH agar plates supplemented with imidacloprid (100 mg L^{-1}).

To evaluate their capacity to remove imidacloprid, 50 μL of each consortia obtained in P4 were inoculated in flasks containing 100 mL sterile BH broth (pH = 6.5) (representing 1.0×10^8 cells mL^{-1} estimated by plate count) with the same conditions of isolation (-N, −C, −CN), supplemented with imidacloprid (50 mg kg^{-1}). Uninoculated flasks were employed as abiotic controls. An analogous set of flasks was used to evaluate the cross-removal of thiamethoxam alone (50 mg kg^{-1}) and in combination with imidacloprid. After incubating for 31 d in the dark (28 °C, 160 rpm), the remaining concentration of neonicotinoids was determined by LC-MS/MS (Section 2.3.2). Cross-degradation of acetamiprid (alone) was determined in a similar assay employing the two consortia of best performance, using both BH-N medium and trypticase soy broth (TSB) and a treatment period of 14 d.

2.2.2. Removal of neonicotinoids by microbial consortium at reactor scale

Stirred tank reactors (5 L; 3 L working volume) operating in batch mode were employed for the simultaneous removal of neonicotinoid mixtures, using the consortium of best degrading performance.

The inoculum was prepared in four flasks containing 150 mL BH-N broth (pH = 6.5) supplemented with 50 mg L^{-1} of imidacloprid and 500 μL of microbial consortium (from P4). These cultures were shaken at 160 rpm, at 25 °C for 5 d and employed as inoculum in the batch reactor. The reactor was loaded with 2.7 L of BH-N medium supplemented with a binary mixture of imidacloprid and thiamethoxam (from commercial formulations, 10–15 mg kg^{-1} a.i. each), and 300 mL of inoculum. Another reactor was loaded with 2.7 L of BH-N medium containing a ternary mixture of imidacloprid, thiamethoxam and acetamiprid (from commercial formulations, 10–15 mg kg^{-1} a.i. each) and 300 mL of inoculum. Agitation was done by two Rushton impellers at 250 rpm and air continuously introduced at 0.5 L min^{-1}. All reactors were maintained at 25 °C in darkness; the initial pH value was 6.5; pH was monitored but not controlled during operation. As controls, two additional reactors were loaded with 3 L of BH-N medium containing the respective pesticides and operated under the same conditions; a flask heat-killed control using inactivated biomass was employed to determine adsorption losses. Pesticide concentrations were determined at times 0, 3, 6, 10, 14, 17, 21, 24, 28 and 30 d, using centrifuged and filtered samples as described in section 2.3.2, and ecotoxicological assays were performed at times 0, 14 and 30 d, using centrifuged samples as described in section 2.3.3.

2.3. Analytical procedures

2.3.1. Microbial population analysis: PCR-DGGE, sequencing and phylogenetic studies

Total genomic DNA was obtained from cell pellets of the enrichment passages (from samples of up to 1 mL; 8000 *g*, 5 min) or colonies from solid phase cultures of the isolates using PowerSoil DNA Isolation kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Extracted DNA was quantified and checked for purity (2000c NanoDrop spectrophotometer); the solutions were maintained at −20 °C and used as templates for PCR amplification of the 16S rRNA genes (Internal Transcribed Spacer 2, ITS2, for a yeast strain) for sequencing or degrading gradient gel electrophoresis (DGGE) as described by Castro-Gutiérrez et al. (2016).

DGGE analysis of the PCR products from the enrichment process was performed with a DCode system (Castro-Gutiérrez et al., 2016). PCR products (5 μL) from individual isolates were purified (ExoSAP-IT™ PCR Product Cleanup Reagent USB, CA, USA) and sequenced using a capillary electrophoresis Genetic Analyzer 3130 (Applied Biosystems) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific Inc., Foster City, USA). Contigs were generated using DNAbaser, and multiple aligned by MAFFT 7 CRBC Server (Katoh et al., 2017). Affiliation of the sequences was done by searching close relatives on Blast (Altschul et al., 1990). A phylogenetic tree of the partial 16S rRNA genes from the consortium members was constructed using the eight bacterial isolates from this study and sequences from other imidacloprid degrading bacteria (Kandil et al., 2015; Pandey et al., 2009; Phugare et al., 2013). The analysis was performed employing Mr. Bayes software (Huelsenbeck and Ronquist, 2001), using 5 million generations and a setting burn-in fraction to 0.25. The bayesian information criterion was selected using Mega7 (Tamura et al., 2011). Existing sequences for other bacteria were retrieved from GenBank. The tree was edited with Inkscape 0.92.2.

2.3.2. Pesticide quantification

Analyses of neonicotinoid insecticides (imidacloprid, thiamethoxam, acetamiprid) and the transformation product 6-chloronicotinic acid were performed from centrifuged/filtered samples by LC-MS/MS using ultra high performance liquid chromatography (UPLC-1290 Infinity LC, Agilent Technologies, CA) coupled to a triple quadrupole mass spectrometer (model 6460). Samples were properly diluted with a mixture of water:acetonitrile (1:1 v/v), both acidified with formic acid (0.1% v/v), before injection. Chromatographic separation was done at 40 °C by injecting 6 μL samples in a Poroshell 120 EC-C18 column (100 mm × 2.1 mm i.d., particle size 2.7 μm), and using acidified water (formic acid 0.1% v/v, A) and acidified methanol (formic acid 0.1% v/v, B) as mobile phases. The mobile phase flow was 0.3 mL min^{-1} at the following conditions: 30% B for 3 min, followed by a 15 min linear gradient to 100% B, 4 min at 100% B and 0.1 min gradient back to 30% B, followed by 5 min at initial conditions. Selected transitions, LOD and LOQ for the analytes are shown in Supplementary Material Table S1. For pesticide analyses, flask scale assays were sampled at the moment of spiking and at 14 d or 31 d; bioreactor assays were sampled at times 0, 3, 6, 10, 14, 17, 21, 24, 28 and 30 d.

2.3.3. Ecotoxicological assays during pesticide removal in bioreactors

2.3.3.1. Seed germination tests. The phytotoxicity of the matrix during the reactor treatment was monitored by seed germination tests with lettuce (*Lactuca sativa* var. Georgia) (USEPA, 1996). Relative seed germination (SG), relative root elongation (RE) and germination index (GI) were determined using 10 seeds exposed to 5 mL of biorreactor samples, after 6 d of incubation in darkness at 22 °C (US Department of Agriculture and US Composting Council, 2001). These parameters were determined by comparison to germination controls obtained by exposure to distilled water, and were calculated as described elsewhere (Huete-Soto et al., 2017). The tests were performed in triplicates to samples withdrawn from the bioreactors at times 0, 14 and 30 d.

2.3.3.2. Vibrio fischeri bioluminescence inhibition test. The experimental procedure was based on the ISO 11348-3 protocol (ISO 11348-3:2007, 1998), using the Microtox® M500 bioassay (Rodriguez-Rodriguez et al., 2011). The percentage of luminescence inhibition was determined by comparing the response given by a saline control to that given by the respective diluted sample, after an exposure time of 15 min. The relative concentration of the sample (considering the pure sample as 100%) that causes 50% inhibition was defined as the EC_{50} value. The test was applied to

bioreactor samples withdrawn at times 0, 14 and 30 d.

2.3.3.3. Acute oral test in honeybees. The test based on the oral acute toxicity of pesticides to adult worker honeybees (*Apis mellifera*) was conducted following the OECD protocol (OECD/OCDE, 1998). Healthy adult bees of the same race and similar age were employed; they were provided and collected by a local apiarist. Collected bees were randomly allocated, ten bees per test cage, and deprived of food for 2 h before the beginning of the test. Four doses (corresponding to dilutions of the reactor samples withdrawn at times 0, 14 and 30 d) in a geometric series with a 2 factor and covering the range of estimated LC_{50} (median lethal concentration), were exposed to triplicate cages. Each test group of bees was provided with 200 μL of 50% w/v sucrose solution, containing the sample dilution at the appropriate concentration; four control groups were supplied with only a sucrose solution. Once consumed (within 3–4 h), the feeder was removed from the cage and replaced with a mixture of sucrose and honey as food, provided *ad libitum*. Mortality was recorded daily for at least 72 h and compared with the control. The bees were held in the dark in an experimental room at 25 ± 2 °C and relative humidity 50–70%. The relative concentration of the sample (considering the pure sample as 100%) that causes a mortality of 50% was defined as the LC_{50} value; LC_{50} values were determined as described in section 2.3.3.4.

2.3.3.4. Data analysis. GI values from seed germination tests were calculated using Excel; EC_{50} values from bioluminescence inhibition test were determined using the Microtox ® software; LC_{50} from the acute oral test in honeybees were determined using the binomial probability test on the TOXCALC-Toxicity Data Analysis Software from Tidepool Scientific Software. Ecotoxicological data for each time of treatment in the reactor were compared (with respect to initial values) using two-way ANOVA and Tukey post-hoc tests to determine whether significant detoxification occurred in each treatment.

3. Results and discussion

3.1. Isolation and identification of imidacloprid-degrading consortia and cross-removal of neonicotinoid pesticides

Weekly passages were done during a selective enrichment process to obtain imidacloprid-degrading consortia, using the insecticide either as the only C-source, N-source or N- and C-source. DGGE profiles during the enrichment are shown in Fig. 1; subsequent passages revealed important alterations on the band patterns compared with the ones from the original soil (S0). In every case, the first passage exhibited a diffuse band pattern, similar to that from soil; from the second passage onwards, several bands prominently appeared as many diffuse bands disappeared, as expected in an enrichment process. Final consortia obtained from the fifth passage were employed for the removal of imidacloprid, thiamethoxam and their combination in flask-scale assays (Table 1). Uninoculated controls revealed abiotic losses (25%–30% for each pesticide) after 31 d; by this time, only consortia N1 and N2 (from the enrichments using imidacloprid as the only N-source, BH-N) showed significant elimination of the insecticides. N1 removed both imidacloprid (60.1%) and thiamethoxam (33.4%; values corrected by abiotic losses from the uninoculated control) when they were individually applied; nonetheless, when the pesticides were simultaneously treated, only imidacloprid was significantly removed (57.9%). On the other hand, N2 only removed imidacloprid (94.9% in individual treatment, or 88.6% in a binary mixture with thiamethoxam), though at a higher extent than N1. Interestingly, these results indicate that at least one imidacloprid-degrading consortium is also capable to cross-degrade thiamethoxam.

Several authors report higher removal of neonicotionoid insecticides by degrading isolates when richer media are employed (Anhalt et al., 2007; Shetti and Kaliwal, 2012), reason why an additional flask-scale assay was performed comparing the individual elimination of imidacloprid and acetamiprid in BH-N and TSB, using the degrading consortia N1 and N2. In the case of imidacloprid, higher removal was achieved in BH-N (31.3%–32.7%) than in TSB (14.3%–25.0%) for both consortia. Although with low efficiency, the isolated consortia were also able to transform acetamiprid; however, for this insecticide higher elimination was obtained in TSB (22.9%–26.8%) than in BH-N (17.2%–18.6%). At this point, consortium N1 is proven as able to transform imidacloprid, thiamethoxam and acetamiprid, while the effect of consortium N2 is notable only on imidacloprid and acetamiprid.

Microbial identification was performed for both, N1 and N2. Eight bacterial strains closely related to the genera *Paenibacillus*, *Rhodococcus*, *Microbacterium*, *Kocuria*, *Paraburkholderia* and *Pseudoacidovorax*, and a yeast strain closely related to the genus *Rhodotorula*, were finally identified from these degrading consortia (partial 16S rRNA/ITS2 gene sequences in GenBank; accession numbers KY347020-KY347021, MH243763-MH243767, MH819692), as shown in Table 2. Phylogenetic analysis of the bacterial strains in the consortia, closely related strains and sequences from other imidacloprid-degrading bacteria is displayed as a phylogenetic tree (Fig. 2). Bacterial isolates from this work clustered as Actinobacteria, Firmicutes and Betaproteobacteria. Comparing these organisms to some of the few imidacloprid degrading strains reported in scientific literature, it is clear that the metabolic ability to transform imidacloprid is present in organisms that are taxonomically distant.

Even though degradation capacity was not tested for individual strains in the N1 and N2 consortia, reports of imidacloprid (or other neonicotinoids) degradation for strains belonging to the identified closest genera is limited. In this respect, only in the case of *Rhodococcus*, the strain BCH-2 has been shown to degrade acetamiprid in a cometabolic process mediated by the formation of 6-chloronicotinic acid (Phugare and Jadhav, 2015). Similarly, strains of *Bacillus* (related to *Paenibacillus*) and *Burkholderia* (closely related to *Pseudoburkholderia*) exhibited the capacity to degrade imidacloprid (Akoijam and Singh, 2015; Madhuban et al., 2011; Sharma et al., 2014). Moreover, little is known about the genes and metabolic pathways regarding imidacloprid degradation. For example, *Mycobacterium* sp. strain MK6 uses the pesticide as a sole N-source (Kandil et al., 2015), while *Pseudomonas* sp. 1G (Pandey et al., 2009), and *Klebsiella pneumoniae* strain BCH1 (Phugare et al., 2013) probably use cometabolic pathways in the presence of alternate C-sources. As with other neonicotinoid-degrading microorganisms, further research is needed to describe the degrading pathway utilized by consortia N1 and N2, and the individual contribution of their members in the process. Moreover, future research should apply metagenomic approaches to better describe the degrading consortia and their shifts during different operation conditions.

3.2. Removal of neonicotinoid mixtures at bioreactor scale

Given the broad removal spectrum exhibited by consortium N1 (imidacloprid, thiamethoxam, acetamiprid), it was selected for scaling-up the process, and 5 L-stirred tank reactors were employed for the removal of two different mixtures of neonicotinoid insecticides in batch operation. No significant pesticide abiotic removal was observed in the reactors during the treatment; besides, adsorption losses were mostly negligible, accounting for 7–8% for each insecticide (Fig. S1), suggesting that elimination can

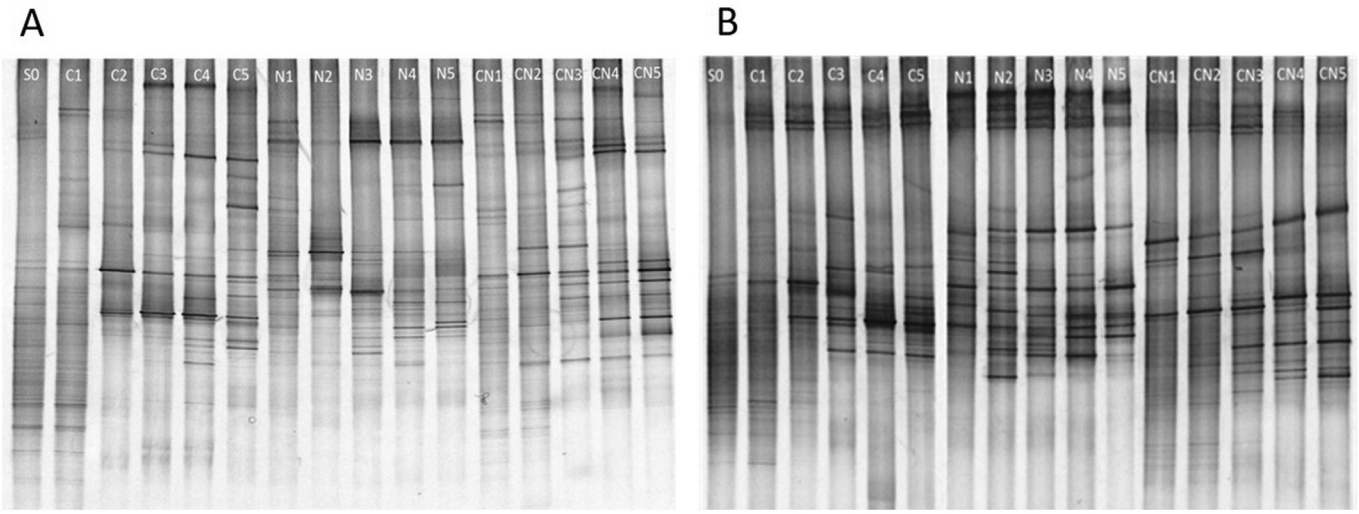

Fig. 1. DGGE profiles of amplicons obtained from the selective enrichment using soils from farms 1 (A) and 2 (B). (S0): soil samples from the farms with a history of imidacloprid application; (C1-C5): band pattern using imidacloprid as the only C-source; (N1-N5) band pattern using imidacloprid as the only N-source; (CN1-CN5) band pattern using imidacloprid as the only C- and N-source. Passages were performed weekly; numbers from 1 to 5 in each set represent the respective passage to fresh medium after 7 d.

Table 1
Residual concentration of neonicotinoids in liquid phase removal assays with different bacterial consortia. Assay 1 included all consortia in simultaneous and individual pesticide elimination; significant elimination values with respect to the uninoculated control are bolded. Assay 2 included only degrading consortia during individual pesticide elimination.

Consortium[a]	Assay 1: Residual pesticide after 31 d (% of initial concentration)[b]		Assay 2: Residual pesticide after 14 d (% of initial concentration)[c]			
	Imidacloprid	Thiamethoxam	Imidacloprid		Acetamiprid	
			BH-N	TSB	BH-N	TSB
Uninoculated control	75.2 (73.1)	72.4 (71.6)	58.8	70.8	71.0	94.7
C1	72.7 (76.0)	75.7 (77.0)	–	–		
N1	**30.0 (30.8)**	**48.2** (67.7)	39.6	53.1	58.8	73.0
CN1	78.8 (76.5)	76.9 (75.7)	–	–		
C2	78.2 (82.0)	77.5 (77.1)	–	–		
N2	**3.8 (8.3)**	76.9 (76.5)	40.4	60.7	57.8	69.3
CN2	75.3 (79.0)	77.2 (75.2)	–	–		

[a] C, consortia from BH-C; N, consortia from BH-N; CN, consortia from BH-CN; numbers 1 and 2 refer to the different soil samples used as inoculum.
[b] Values correspond to individual removal; values in parenthesis correspond to simultaneous treatment of both insecticides.
[c] Culture media: BH-N, Bushnell-Haas lacking N source; TSB, trypticase soy broth.

Table 2
Closest strains on the GenBank database for the isolates from the imidacloprid degrading consortia, based on 16S rRNA or ITS2 sequences.

Consortium	Isolate	Closest type strain (GenBank)	Accession No. of closest type strain	% Similarity (No. of bases compared)
N1	CN2MF2	*Kocuria rhizophila*	Y16264	98.92 (1383)
	CN2MF3	*Paraburkholderia phymatum* STM815	CP001043	99.32 (1183)
	CN2MF4	*Rhodotorula toruloides* culture CBS:12015	KY104915.1	99.84 (630)
	CN2MF6	*Paenibacillus odorifer* strain DSM 15391	CP009428	98.39 (1243)
N2	CN5MF1A	*Paenibacillus odorifer* strain DSM 15392	CP009428	96.77 (1425)
	CN5MF1B	*Rhodococcus aetherovorans* strain 10bc312	AF447391	96.84 (1328)
	CN5MF2	*Microbacterium binotii* strain CIP 101303	EF567306	98.08 (1357)
	CN5MF3	*Microbacterium binotii* strain CIP 101304	EF567307	99.19 (1365)
	CN5MF6	*Pseudoacidovorax intermedius* strain CC-21	EF469609	98.63 (1390)

be ascribed to the effect of the viable microbial biomass. In fact, fast growth of the consortium was observed in the medium in the presence of the three insecticides, increasing from 2.5×10^5 CFU/L at the moment of inoculation, to 9.3×10^{10} CFU/L, after 3 d of treatment (Fig. S2). A first batch assay containing the binary mixture imidacloprid/thiamethoxam was able to completely remove imidacloprid after 10 d (>99% removal in 6 d) and 87.6% thiamethoxam after 30 d (Fig. 3). A second batch assay simultaneously treating the ternary mixture imidacloprid/thiamethoxam/acetamiprid completely removed imidacloprid after 17 d, though at slower rates than in the reactor containing the binary mixture; acetamiprid was also removed (99.9%) after 28 d (Fig. 4; Fig. S3). On the other hand, the elimination of thiamethoxam seemed unaffected by the presence of the additional pesticide, and 87.3% was removed after 30 d. Remarkably, 6-chloronicotinic acid, a common transformation product from first generation neonicotinods (imidacloprid, acetamiprid) (Hao et al., 2016), was not detected in the reactors, which could indicate either that it was not produced, or

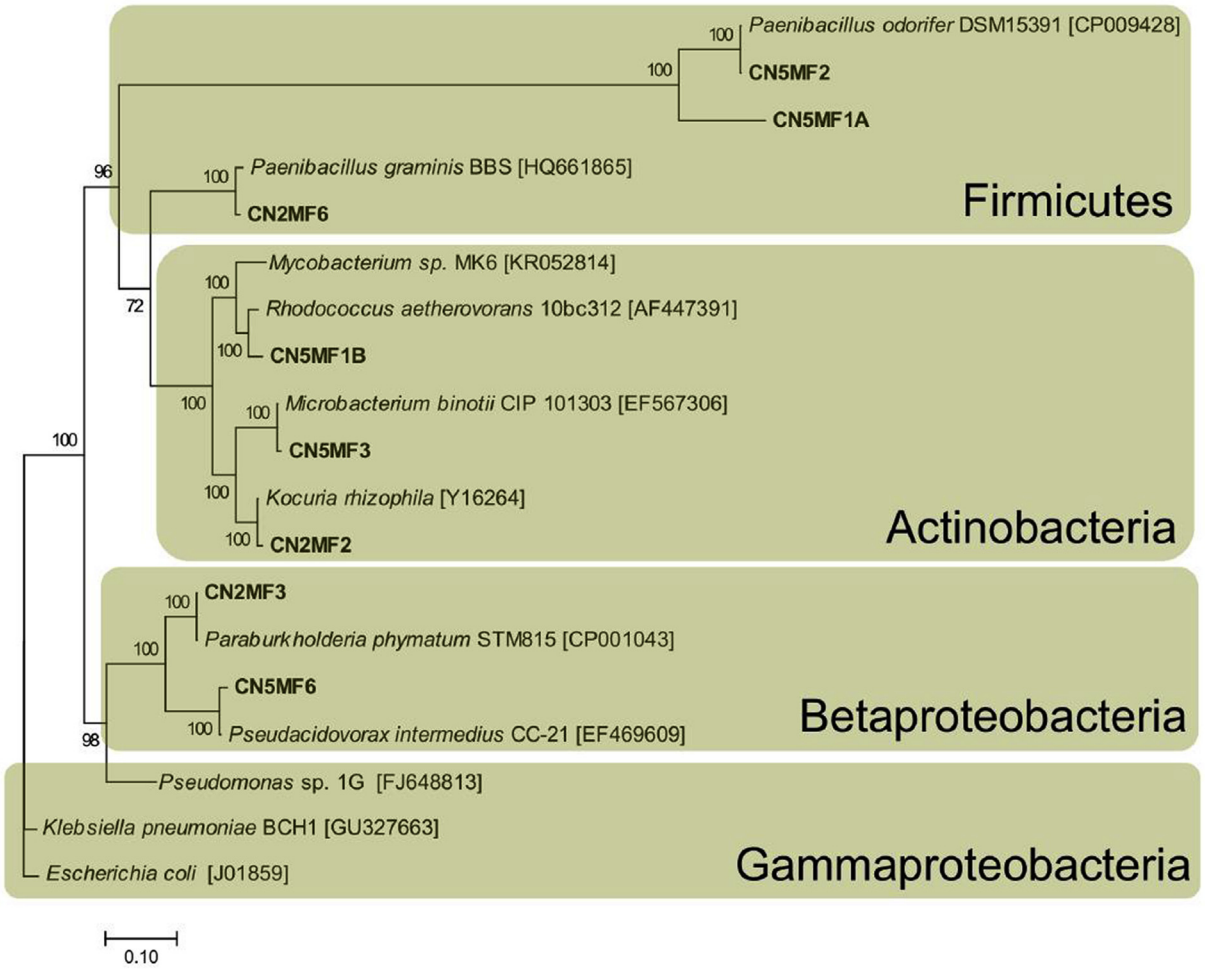

Fig. 2. Bayesian phylogeny of bacterial isolates from the imidacloprid degrading consortium N1 and N2. The phylogenetic tree was constructed using 16S rRNA partial gene sequences. Scale bar represents 0.10 substitutions per nucleotide position. The numbers above the branches represent their Bayesian-calculated posterior probabilities. Existing sequences for other imidacloprid-degrading bacteria *Mycobacterium* sp. MK6, *Pseudomonas* sp. 1G, *Klebsiella pneumoniae* BCH1(Pandey et al., 2009; Phugare et al., 2013; Kandil et al., 2015) were retrieved from GenBank.

that its elimination rate was faster than its production rate, thus never reaching a detectable concentration in this matrix. This metabolite is highly relevant in soil (Lewis et al., 2016); moreover, it has been detected as a major product from imidacloprid and acetamiprid transformation by degrading strains of *Mycobacterium* sp (Kandil et al., 2015) and *Rhodococcus* sp (Phugare and Jadhav, 2015), respectively. Summarizing, the simultaneous treatment revealed that all neonicotinoids tested were effectively degraded by the microbial consortium; imidacloprid was in both systems the compound of fastest elimination.

Nonetheless, an apparent lag phase of around 10 days was detectable for imidacloprid transformation when co-treated with thiamethoxam and acetamiprid (Fig. 4), and not in the binary mixture with thiamethoxam. Such lag phase delayed, but did not inhibit, the complete removal of this insecticide. Acetamiprid, a smaller molecule of shorter half-lives in the environment, was removed slower than imidacloprid but faster than thiamethoxam (Lewis et al., 2016); however this is not surprising considering the origin and enrichment procedure used to obtain the consortium (only with imidacloprid). Lastly, thiamethoxam exhibited the lowest removal rates, and around 13% of the original concentration remained by the end of the treatment. Overall, neonicotinoid removal totaled 95.8% and 94.4% in the binary and ternary mixtures after the treatment period of 30 d.

Neonicotinoid degradation was more efficiently achieved in this study by consortium N1 at reactor-scale than at flask-scale (Table 1). Conditions in the reactor, including forced aeration and better oxygen mass transfer could favor the accelerated pesticide elimination. The reactor performance also surpassed the efficiency observed in several studies that employed isolated strains at flask-scale. For instance, *Pseudomonas* sp. 1G. decreased thiamethoxam and imidacloprid levels by 70% after 14 d, at initial concentrations of 50 mg L^{-1} (Pandey et al., 2009). Other researchers (Wang et al., 2011) demonstrated the transformation of acetamiprid (72/40%) and imidacloprid (11/23%) by *Acinetobacter* sp. and *Sphingomonas*, respectively, after 3 d at initial concentrations of 500 mg L^{-1}. The yeast *Rhodotorula mucilaginosa* removed 93.5% of 500 mg L^{-1} acetamiprid after 14 d and 59.9% of 200 mg L^{-1} thiacloprid after 20 d (Dai et al., 2010). As these reports were performed at flask-scale and for individual compounds, the use of a reactor for the simultaneous treatment of several insecticides proposed in this work represents an important contribution in the scaling-up of processes for the elimination of neonicotinoids.

Microbial consortia have often proven to be more efficient than isolated strains for bioremediation of recalcitrant compounds. Their biodiversity increases the occurrence of metabolic pathways that involve stepwise transformations by microbial consortia members (Smith et al., 2005). Moreover, the use of individual isolates from a

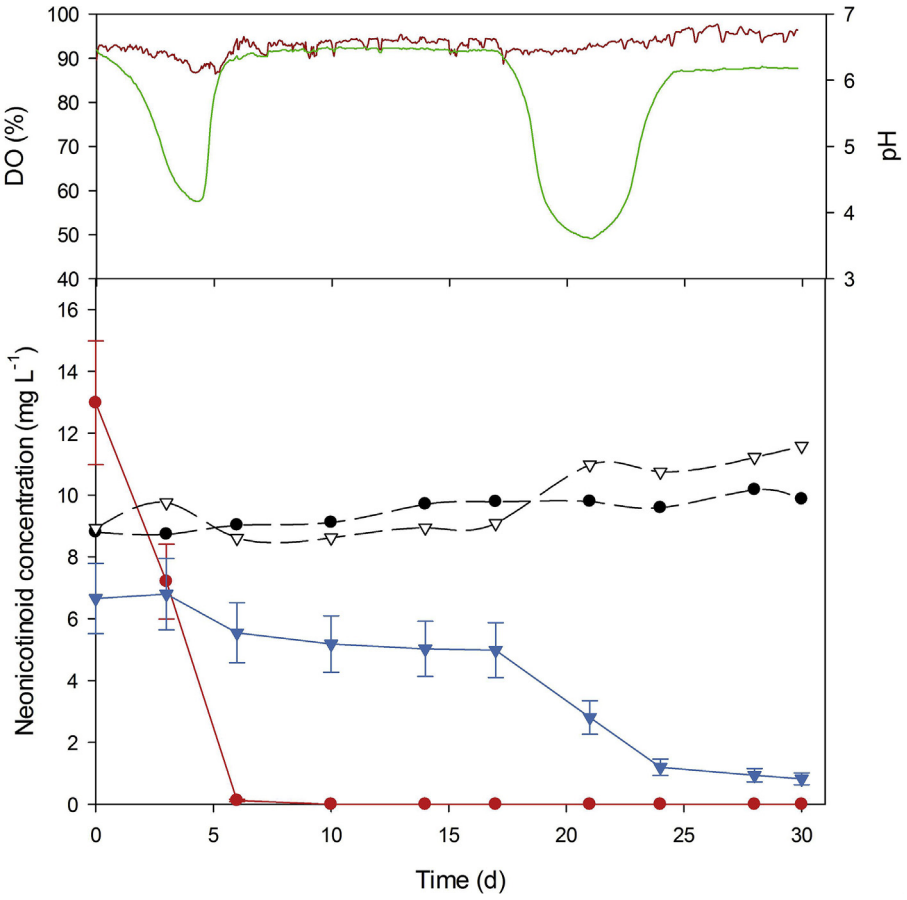

Fig. 3. Removal profiles during the simultaneous treatment of imidacloprid (red circles) and thiamethoxam (blue triangles) by the microbial consortium (N1) in a batch STBR. Controls in uninoculated reactors are shown in dotted lines: imidacloprid (black circles) and thiamethoxam (blank triangles). Dissolved oxygen (DO, dark red) and pH (green) variations are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

single-degrading consortium has failed to remove the pesticides transformed by the whole consortium (Castro-Gutiérrez et al., 2016); in addition, the neonicotinoid cross-degrading capacity exhibited by the consortium could be absent from individual isolates. These facts support the use of consortia, rather than individual strains, in bioremediation strategies.

3.3. *Ecotoxicological variations during the removal of neonicotinoid mixtures at bioreactor scale*

Considering the deleterious effect of neonicotinoids on non-target organisms, and in particular on pollinators, the transformation of neonicotinoids is not enough to guarantee the eco-feasibility of the process, as the treatment might result in the formation of toxic transformation products. In this respect, ecotoxicological assays were performed to estimate the detoxification potential of the reactor treatment. Seed germination tests using lettuce as a biomarker of phytotoxicity (Fig. 5A), showed initial GI values of 74.4% and 67.4% for the reactor treating the binary and ternary mixtures, respectively. Even though time-related differences were not significant in every case, detoxification (linked to increased GI values) was observed, particularly in the reactor used for the treatment of two insecticides, for which GI was close to 100% after 14 d. GI values (and in general apparent detoxification) were lower in the system containing the ternary mixture of insecticides. Hence, the additional presence of acetamiprid and the higher residual concentration of thiamethoxam in the latter reactor may be the causes of these results, given that in this system a higher diversity of transformation products is expected to be formed if compared to the reactor containing the binary mixture; the interactions among these transformation products and residual parent compounds create a unique “toxicological environment” in each condition.

Bioluminescence inhibition assays with *V. fischeri* revealed clear detoxification from the binary mixture treatment, dramatically increasing the EC_{50} value after 14 d of treatment (Fig. 5B); expressed as toxicity units (TU), the decrease in toxicity was >99.99%. On the contrary, in the treatment of the ternary mixture no detoxification was estimated, basically due to the unexpected relatively low toxicity determined at the beginning of the reactor treatment (time zero), which was similar to the EC_{50} at the end of the treatment. Similar toxicity was expected at initial times in both reactors (or even higher in the one containing the ternary mixture); however, initial concentration of pesticides was higher in the ternary vs the binary mixture reactor (25 vs 19 mg/L), and the initial amount of (detected) imidacloprid was markedly higher in the binary mixture. In the case that imidacloprid is the most toxic (among the employed neonicotinoids) to this specific benchmark organism (for which ecotoxicological data of neonicotinoids is scarce), then it is possible to obtain a higher toxicity in the binary mixture reactor. On the other hand, potential antagonist or synergistic effects could take place in the interactions between the neonicotinoids (and their transformation products) in such a complex chemical environment. Therefore, with our data we cannot rule out the

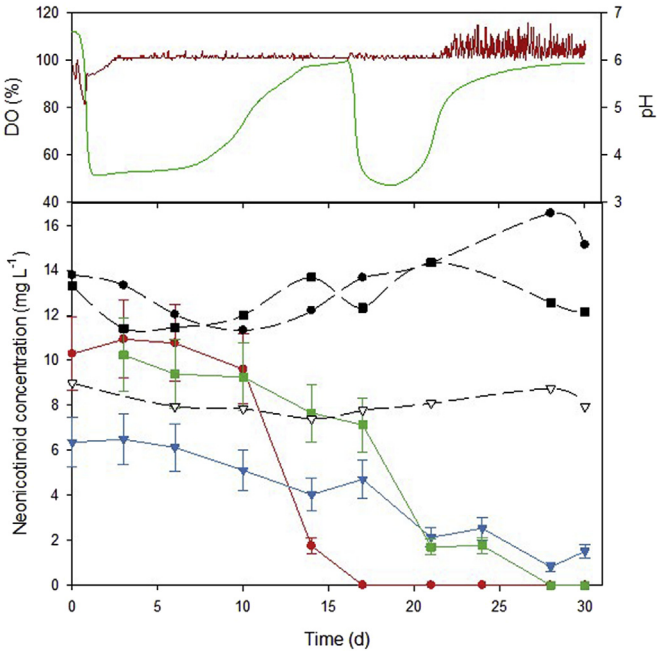

Fig. 4. Removal profiles during the simultaneous treatment of imidacloprid (red circles), thiamethoxam (blue triangles) and acetamiprid (green squares) by the microbial consortium (N1) in a batch STBR. Controls in uninoculated reactors are shown in dotted lines: imidacloprid (black circles), thiamethoxam (blank triangles) and acetamiprid (black squares). Dissolved oxygen (DO, dark red) and pH (green) variations are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

possibility that the addition of acetamiprid exerts some antagonistic effect in combination with imidacloprid/thiamethoxam, thus resulting in a lower initial toxicity detected in the Microtox test.

Due to the current concern regarding toxicity of neonicotinoids towards pollinator insects, acute oral tests in honeybees were performed to monitor their removal in the bioreactor (Fig. 5C). Most of the toxicity is expected to be ascribed to imidacloprid and thiamethoxam, as they have the highest individual toxicity towards honeybees (oral acute test, LC_{50} 0.0037 and 0.005 μg bee^{-1}, respectively (Lewis et al., 2016)); acetamiprid exhibits lower individual toxicity (LC_{50} 14.53 μg bee^{-1} (Lewis et al., 2016)) nonetheless interactions among the three pesticides and their transformation products during the treatment process make it difficult to predict the ecotoxicological outcome. As expected, the highest toxicity values were recorded at the beginning of the treatment (although higher in the system containing the binary mixture, which can be explained by its higher initial concentration of (detected) imidacloprid, the most toxic towards honeybees). Overall, the process was able to decrease the toxicity of the pesticide mixtures, correlating with the elimination of the insecticides. Nonetheless, detoxification was faster in the reactor treating the binary mixture of neonicotinoids, which exhibited lower residual toxicity values by the end of the treatment. Expressed as toxicity units, detoxification reached 75.0% after 14 d and 93.8% after 31 d in this system. On the other hand, in the reactor containing the ternary mixture, detoxification was apparent only at the end of the process, reaching 81.8% after 31 d.

As described for seed germination tests, the presence of an additional active ingredient (acetamiprid), and its slower elimination compared to imidacloprid, might explain the findings in the acute oral test in honeybees. Phugare et al. (2013) demonstrated a decrease in the oxidative stress (lipid peroxidation, protein oxidation, antioxidant enzyme production) and DNA damage in

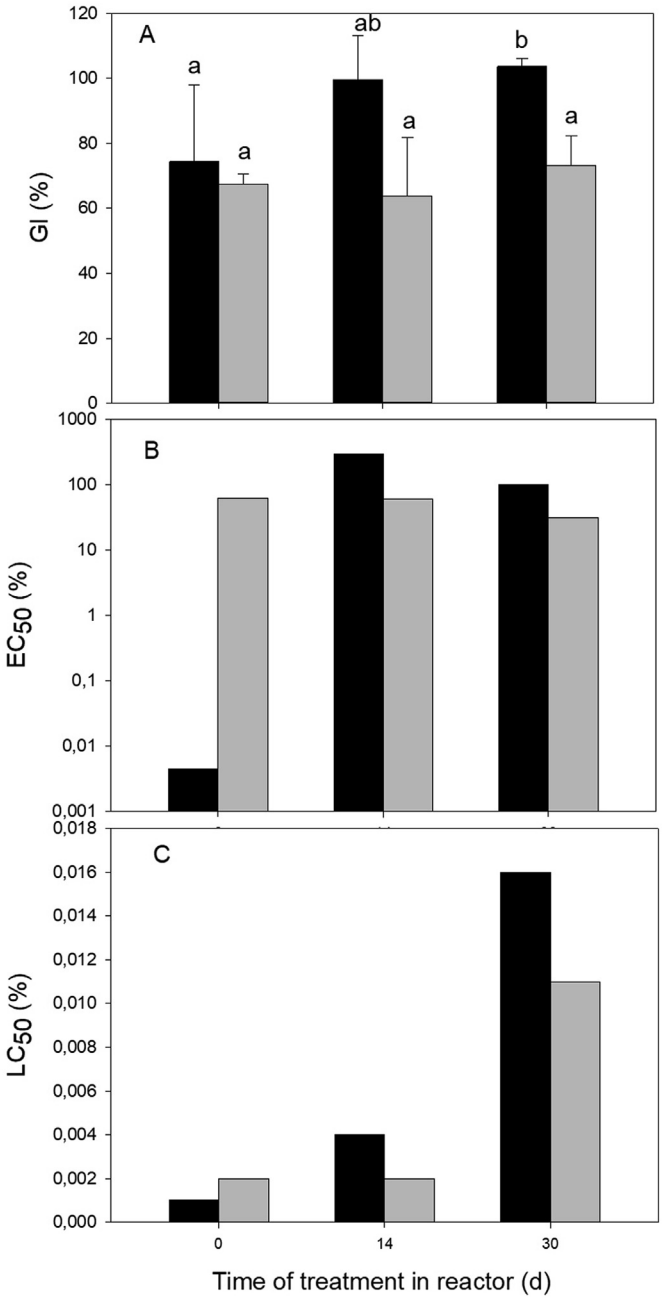

Fig. 5. Variations in the ecotoxicity of the synthetic neonicotinoid-containing wastewater throughout their treatment by the microbial consortium N1 in a batch STBR. Biomarkers: (A) Seed germination test with lettuce (*L. sativa*), toxicity expressed as germination index, GI (%). (B) *V. fischeri* bioluminescence inhibition test, toxicity expressed as EC_{50}, the relative concentration of the sample (considering the pure sample as 100%) that causes 50% inhibition. (C) Acute oral test in honeybees, toxicity expressed as LC_{50}, the relative concentration of the sample (considering the pure sample as 100%) that causes a mortality of 50% (C). Simultaneous removal of imidacloprid and thiamethoxam (black bars); simultaneous removal of imidacloprid, thiamethoxam and acetamiprid (grey bars). Different letters indicate statistically significant differences in the toxicity ($p < 0.05$).

silkworms after the biological treatment of imidacloprid with a *Klebsiella pneumoniae* strain, compared to the effect of the untreated insecticide. However, the values of these parameters were still altered with respect to controls.

Even though clear and significant detoxification was achieved, the residual toxicity could still exert adverse effects on pollinators.

Also, considering that after 30 d thiamethoxam is still present in both systems at concentrations of around 1 mg L^{-1}, longer treatment periods should be further evaluated. Likewise, complementary treatments are strongly recommended to achieve a more complete detoxification of neonicotinoid-containing wastewater. The use of several biomarkers should be employed in neonicotinoid removal assays to attain a better estimation of the ecotoxicological effects of the treatment residues; to the knowledge of the authors, this work represents the first approach to monitor the biological degradation of neonicotinoids with multiple ecotoxicological assays, including honey bees as biomarkers.

4. Conclusions

A selective enrichment approach (from imidacloprid primed soil) permitted the isolation of microbial degrading consortia with the ability to remove imidacloprid by a cometabolic process. Members of the consortia have not been previously reported as imidacloprid-degrading species; however, the specific role of individual strains needs further studies. The most efficient degrading consortium was also able to cross-remove the neonicotinoid insecticides thiamethoxam and acetamiprid, though less efficiently than imidacloprid. Combinations of neonicotinoids (namely: imidacloprid + thiamethoxam or imidacloprid + thiamethoxam + acetamiprid) were eliminated from synthetic wastewater when the consortium was applied at a stirred tank bioreactor scale, with rates imidacloprid > acetamiprid > thiamethoxam; nonetheless, in this system the imidacloprid removal rate was negatively affected by the most complex mixture of pesticides. Considering that according to different biomarkers including honeybees, the residual ecotoxicity of the wastewater was partially decreased by the reactor treatment, this approach seems a promising strategy to remove the highly toxic and persistent neonicotinoids from agricultural wastewaters. Further research should be focused on optimizing the process to achieve higher detoxification levels, and on adapting it to feasible conditions for farms of different sizes and devoted to the production of diverse crops.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.07.004.

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