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Diuron degradation by bacteria from soil of sugarcane crops

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Abstract

The isolation of microorganisms from soil impacted by xenobiotic chemicals and exposing them in the laboratory to the contaminant can provide important information about their response to the contaminants. The purpose of this study was to isolate bacteria from soil with historical application of herbicides and to evaluate their potential to degrade diuron. The isolation media contained either glucose or diuron as carbon source. A total of 400 bacteria were isolated, with 68% being Gram-positive and 32% Gram-negative. Most isolates showed potential to degrade between 10 and 30% diuron after five days of cultivation; however *Stenotrophomonas acidophila* TD4.7 and *Bacillus cereus* TD4.31 were able to degrade 87% and 68%, respectively. The degradation of diuron resulted in the formation of the metabolites DCPMU, DCPU, DCA, 3,4-CAC, 4-CA, 4-CAC and aniline. Based on these results it was proposed that *Pseudomonas aeruginosa* TD2.3, *Stenotrophomonas acidaminiphila* TD4.7, *B. cereus* TD4.31 and *Alcaligenes faecalis* TG 4.48, act on 3,4-DCA and 4-CA by alkylation and dealkylation while *Micrococcus luteus* and *Achromobacter* sp follow dehalogenation directly to aniline. Growth on aniline as sole carbon source demonstrates the capacity of strains to open the aromatic ring. In conclusion, the results show that

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the role of microorganisms in the degradation of xenobiotics in the environment depends on their own metabolism and also on their synergistic interactions.

Keywords: Microbiology, Environmental sciences

1. Introduction

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, DIU) is a herbicide used in a range of crops and gardening and other non-crop areas to control the development of weeds and has also been used as an algaecide in antifouling paints. The widespread use of these herbicides has, together with its persistence, resulted in it becoming a common pollutant in both soil and water (Hasan et al., 2012; Giacomazzi and Cochet 2004). Long term xenobiotic contamination of an area results in a selection and adaptation of the microbial population for tolerance and degradation (Sørensen et al., 2008). However, DIU mineralization occurs through a cooperative metabolism among microbial populations (Devers-Lamrani et al., 2014; Villaverde et al., 2017) and primarily depends on the genetic pool of the innate microbiota and fauna (Rippy et al., 2017; Ellegaard-Jensen et al., 2014).

In environments contaminated with diuron, the degradation products methyl(3,4-dichlorophenyl-methylurea, DCPMU) and dimethyl(3,4-dichlorophenyl-methylurea, DCPU) have been detected (Tixier et al., 2002), in addition to aniline and its chlorinated derivatives (3,4-dichoroaniline and 4-chloroaniline). The formation of DCPMDU (3,4-dichlorophenylmethylideneurea) as an intermediate between DCPMU and DCPU was reported by Ellegaard-Jensen et al., 2013. These metabolites indicate that the diuron degradation usually proceeds by successive N-demethylation and amide bond cleavage involving demethylase and amidase reactions (Dalton et al., 1966; Tillmanns et al., 1978; Sørensen et al., 2008). A demethylase associated to cytochrome P450 was detected in *Pseudomonas* (Chakraborty et al., 2005). The biodegradation pathway of the metabolite 3,4-DCA consists of dechlorination resulting in the formation of 4-CA and aniline, which undergoes deamination to generate catechol with subsequent ring cleavage (Breugelmans et al., 2010). Hongsawat and Vangnai (2011) proposed a second pathway for 3,4-DCA degradation proceeding by 4-amine-2-chlorophenol formation, followed by formation of 4-chlorocatecol and 3-chloro-*cis*-*cis*-muconic acid.

Two genes coding for enzymes involved in the transformation of dimethyl-substituted phenylureas into their aniline derivatives have been identified. The phenylurea hydrolase A gene (puhA) was identified in *A. globiformis* strain D47 (Turnbull et al., 2001) and the phenylurea hydrolase B gene (puhB) was characterized in *Mycobacterium brisbanense* JK1 (Khurana et al., 2009). Through a proteomic approach several copies of the aniline dioxygenase (AD) gene cluster, responsible for 3,4-DCA degradation and expressed in the presence of 3,4-DCA,

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were identified in strains *Variovorax* sp WDL1 and SR16 (Breugelmans et al., 2010).

The dechorination mechanism has not been described for diuron but dehalogenation of halogenated aromatic substrates is known and follows different pathways depending on whether the conditions are aerobic or anaerobic (Häggblom and Bossert, 2003). Chlorine elimination may occur as an initial step via reductive, hydrolytic or oxygenolytic mechanisms, or after cleavage of the aromatic ring at a later stage of metabolism (Kim et al., 2009). Cleavage of carbon-chlorine bonds before cleavage of the aromatic ring can be catalyzed by dioxygenases that incorporate two oxygen atoms accompanied by dehydrodehalogenation forming catechol intermediates. Reductive dehalogenation involves the tetrachlorohydroquinone reductive dehalogenases that catalyzes the reductive displacement of chlorine from aromatic ring by a glutathione-dependent reaction involving CoA-SH. The system 4-chlorobenzoyl coenzyme A dehalogenase uses a hydrolytic mechanism to cleave the carbon-halogen bond of 4-chlorobenzoate as a CoA derivative. These enzyme systems have been described for a number of bacteria. *Pseudomonas fluorescens* 26 K removed the chlorine substituent from 3,4-DCA forming 3-chloro-4-hydroxylaniline by action of chlorocatechol-2-3-doxygenase (Isin and Guengerich 2007; Janssen et al., 2001; Seeger et al., 1997). Dechlorination coupled to ring cleavage was observed in *Pseudomonas putida* that degraded 3,4-DCA to 4,5-dichlorocatechol with *ortho* ring cleavage (Breugelmans et al., 2010).

The aim of this study was to characterize bacteria strains isolated from soil of sugarcane crops which had undergone frequent diuron application in order to assess their potential to degrade this herbicide and to identify the biodegradation pathways employed by these bacteria.

2. Results

2.1. Bacteria isolation

A total of 400 bacterial strains were isolated from the soil samples, with equal numbers isolated from medium with glucose and medium with both diuron and glucose as the carbon source (200 each). For example, in the dilution of 10^{-6}, 65 to 57 colony forming units (CFUs) were observed in the entire sample, equivalent to 1.2×10^{8} bacteria per gram of the soil. However, a higher morphological diversity was observed among bacteria with glucose than with diuron, suggesting that the diuron had a selective effect on tolerant/sensitive bacteria. Of the 400 isolated strains, 68% were Gram-positive bacteria (GP) and 32% Gram-negative (GN) (Table 1).

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Table 1. Characteristics of diuron-degrading bacteria isolated with different media.

	Medium with glucose	Medium with diuron	Total
Number of strains isolated	200 (50%)	200 (50%)	400 (100%)
Gram-positive bacteria	128 (64%)	146 (73%)	274 (68.5%)
Gram-negative bacteria	72.0 (36%)	54.0 (27%)	126 (31.5%)
Bacteria that degrade up to 19% (9.5 mg L^{-1}) of the diuron	163 (40.7%)	108 (27%)	271 (68%)
Bacteria that degrade more than 20% (10 mg L^{-1}) of the diuron	21.0 (7.7%)	24.0 (19%)	45.0 (11%)
Metabolites released from diuron degradation			
DCPMU (mg L^{-1})	0.16 up to 0.94		
DCPU (mg L^{-1})	0.12 up to 0.67	0.13 up to 0.53	
DCA (mg L^{-1})	0.28 up to 0.80	0.36 up to 0.42	

The ability of the bacterial strains to degrade diuron was determined by analysis of residual diuron in the culture medium of the 400 strains compared to that in the abiotic control. The data in Table 1 show that 45% of the total bacteria (GP and GN) isolated in the medium containing only glucose showed some ability to degrade diuron against 33% isolated in medium with diuron and glucose as carbon sources. However, the best degradation (degradation higher than 10 mg L^{-1} in 120 h of cultivation) was obtained with 19% of GN against 7.7% of GP.

Among the 10 strains that showed the highest degradation rates, 8 were identified as GN species (Table 2). These strains were cultivated again in diuron-containing medium to confirm their ability to degrade diuron and to identify intermediates formed during degradation. The results (Table 2) corroborate those from the first experiment and allowed the identification and/or quantification of DCPMU, DCPU and 3,4-DCA. In some cultures, the concentrations of metabolites were either too low to allow quantification or were below the detection limit (Table 2).

2.2. Study of diuron degradation pathway

The identification of the metabolites listed in Table 2 in the cultures of the best diuron-degrading strains indicates that diuron degradation by these strains begins with a series of N-demethylation reactions releasing in turn 3,4-dichlorophenyl-methylurea (DCPMU), 3,4-dichlorophenyllurea (DCPU) followed by amide bond cleavage to generate 3,4 dichloroaniline (3,4-DCA). There were, however, differences between the strains in the type and concentrations of metabolites identified.

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Table 2. Strains showing highest diuron degradation rates and metabolites of diuron identified by HPLC.

Strain	ID NCBI*	Diuron degradation (%)	Rate of degradation (µg day $^{-1}$)	Metabolites (µg.L^{-1})		
Gram-negative				DCPMU	DCPU	3.4-DCA
Stenotrophomonas acidaminiphila TD4.7	MG214516	84	90	+	nd	0.10
Delftia sp. TD3.31	MG214518	47	45	nd	0.07	0.10
Alcaligenes faecalis TG4.48	MG214519	35	34	+	+	+
Ochrobactrum intermedium G3.48	MG214517	33	33	nd	0.16	nd
Achromobacter sp. TD2.18	MG214481	32	32	0.18	nd	0.09
Pseudomonas aeruginosa TD2.3	MG214521	29	29	nd	nd	0.63
Enterobacter sp. TG2.31	MG214482	15	14	0.19	0.23	0.20
Gram-positive						
Bacillus cereus TD4.31	MG214520	68	56	nd	nd	nd
Micrococcus luteus TG3.30	MG214408	29	29	nd	0.06	nd
Leucobacter sp. TG3.14	MG214474	11	11	0.25	0.36	1.13

+ = quantitative analysis by MS/MS; nd = not detected; Diuron concentration: 50 mg L^{-1}; * genBank.

Considering the common degradation route from diuron to 3,4-DCA for all strains, the bacteria were cultivated for five days in media containing 10 mg L^{-1} of 3,4-DCA instead of diuron, in order to evaluate their ability to degrade this metabolite and identify its degradation pathway. All the bacteria were able to dissipate the 3,4-DCA more efficiently than diuron (12 to 95%), except for *Stenotrophomonas acidaminiphila* TD4.7 that was able to degrade 84% of diuron (Table 2) and only 20% of the 3,4-DCA in the culture medium (Fig. 1). *Alcaligenes faecalis* TG4.48 and *Achromobacter* sp TD2.18 showed the highest extent of dissipation of 3,4-DCA (100 and 80%, respectively).

Analysis of the culture media of *Pseudomonas aeruginosa* TD2.3, *S. acidaminiphila* TD4.7 and *Bacillus cereus* TD4.31 by LC-MS and GC-MS revealed the presence of 3,4-dichloroacetanilide (3,4-CAC) (Fig. 2a), produced by the acetylation of the 3,4-DCA molecule. The metabolite 4-chloroaniline (4-CA) (Fig. 2b), derived from the dehalogenation of 3,4-DCA, was detected in culture media of *Micrococcus luteus* TG3., *A. faecalis* TG4.48 and *Achromobacter* sp. TD2.18. The strains *Enterobacter* sp TG 2.31 and *Leucobacter* sp TG3.14 were not studied in this way because of their low extent of diuron degradation.

In order to investigate the biodegradation pathways of 3,4-CAC and 4-CA, these intermediates were added to the culture media of the eight bacteria, under the same conditions as described for diuron. The removal of 4-CA ranged from 0.9 to 53% (Fig. 3) and coincided with the potential for degradation of 3,4-DCA for *A. faecalis* TG4.48, *P. aeruginosa* TD2.18 and *B. cereus* TD4.31. *Achromobacter* sp TD2.18

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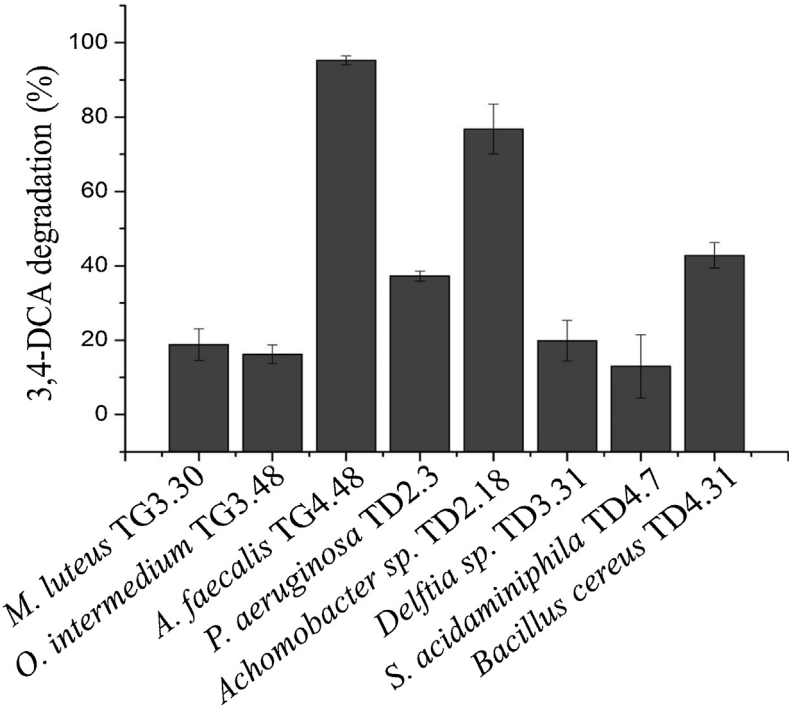

Fig. 1. Degradation of 3,4-DCA by bacteria in 5 days of cultivation (initial concentration = 10 mg. L^{-1}).

was able to degrade 3,4-DCA but not 4-CA. On the other hand, *M. luteus* TD3.30 degraded 4-DCA but very little 3,4-DCA. *O. intermedium* TG 3.48 did not degrade 4-CA and *Delftia* sp. TD3.31 and *S acidaminiphila* TD 4.7 showed low degradation of this compound.

The bacteria *M. luteus* TG 3.30 and *Achromobacte*r sp TD 3.31 were able to dehalogenate 4-CA to aniline (Fig. 4). Two different peaks were detected in chromatograms from the media of *P. aeruginosa* TD2.3, *S. acidaminiphila* TD4.7, *B. cer*eus TD4.31 and *A. faecalis* TG 4.48 after cultivation: aniline, resulting from the second dehalogenation of 4-CA, and 4-chloroacetanilide (4-CAC), indicating that these bacteria also acetylated 4-CA. Since this bacteria also produced an acetylated derivative from 3,4-DCA, the acetylated metabolite 3,4-DAC was used in the culture media and deacetylation of 3,4-DAC was observed (Fig. 4).

The strains *O. intermedium* TG3.48, *P aeruginosa* TD 3.4 and *Achromobacter* sp TD 2.28 were able to grow when aniline was used as the sole carbon source (10 mg L^{-1}), demonstrating the ability of these bacteria to cleavage the aromatic ring (Fig. 5).

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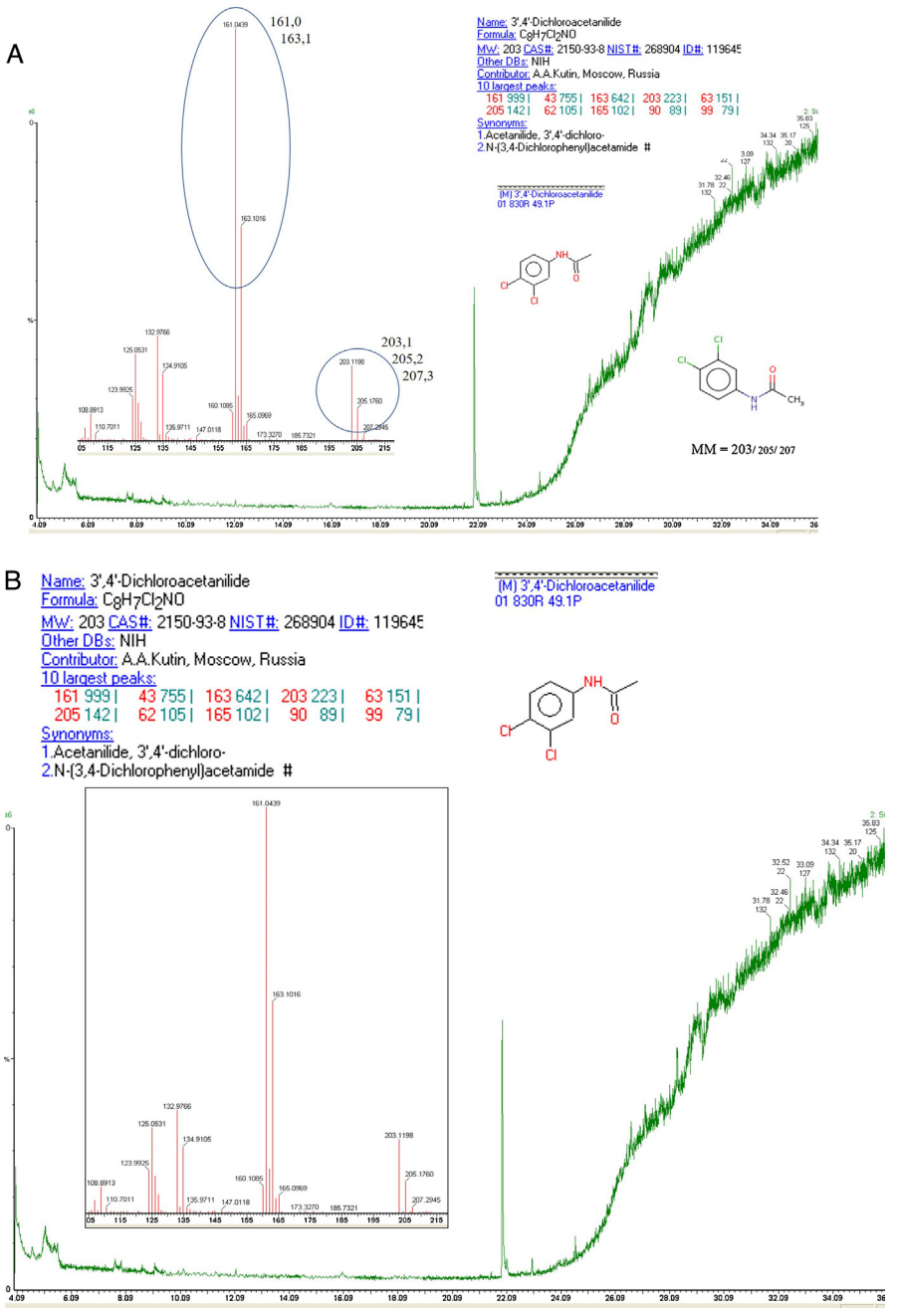

Fig. 2. Overlapping HPLC chromatograms from 8 bacteria after 5 days of cultivation in 10 mg.L^{-1} of 3,4- DCA (a). Each identified and non-identified metabolite were showed with its respective retention time. All peaks, relating to the components of the culture media, were eluted by 3 min and were deleted. (b) GC-MS chromatogram and mass spectrum for the detection of the metabolite 3,4-dichloroacetanilide (3,4-CAC).

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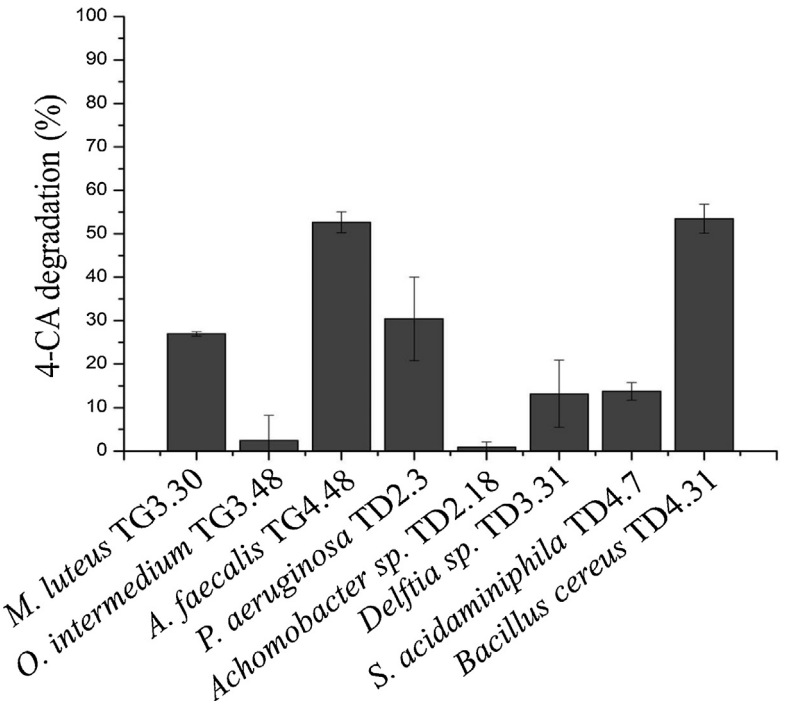

Fig. 3. Degradation of 4-CA by bacteria in five days of cultivation (initial concentration = 10 mg.L^{-1}).

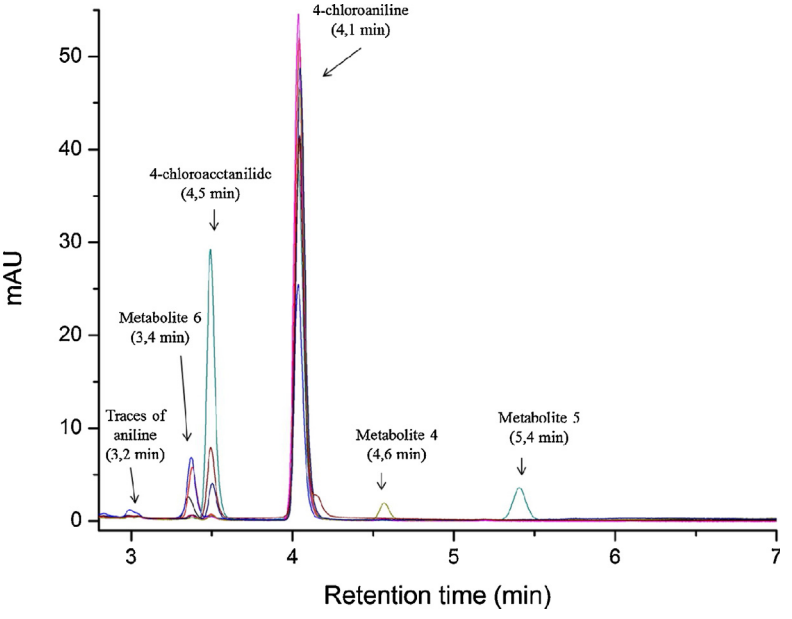

Fig. 4. Overlapping HPLC chromatograms from 8 bacteria after 5 days of cultivation in 10 mg. L^{-1} of 4-chloroaniline.Each identified and non-identified metabolite is shown with its respective retention time. All peaks, relating to the components of the culture media were eluted before 3 min and are deleted.

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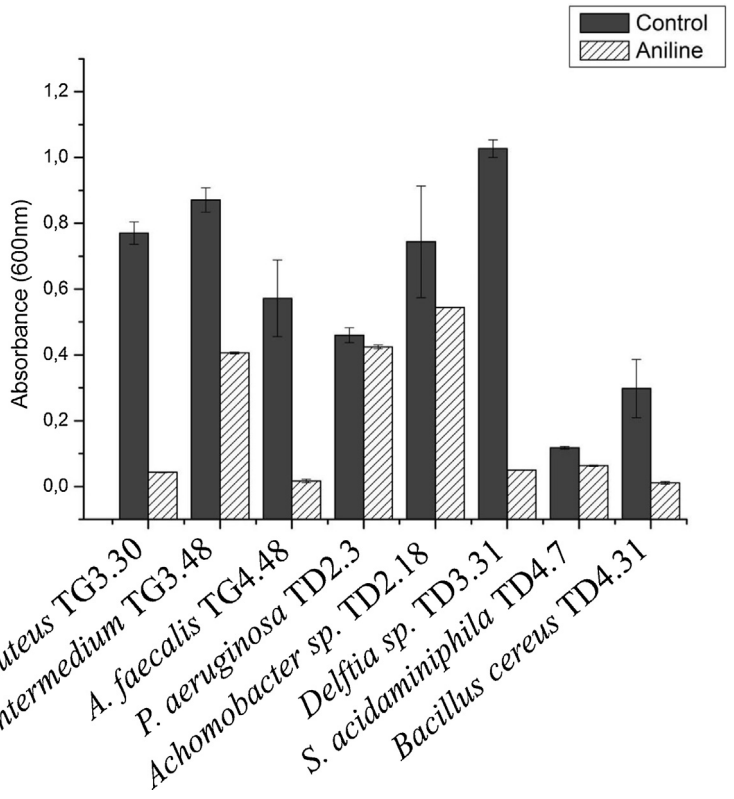

Fig. 5. Growth of bacteria for 5 days in media with glucose (control) or aniline as carbon source (10 mg. L^{-1}).

3. Discussion

The higher number of Gram-positive bacteria isolated from the soil samples is in agreement with previous reports. Due to their type of cell wall and to their endospore forming ability, Gram-positive bacteria are more tolerant to stress compared with the Gram-negatives. The GN bacteria are usually more sensitive to environmental variations such as type and concentration of the carbon source and the presence of xenobiotics (Yang et al., 2000; Banks et al., 2014). There are many diverging data reported about soil microbial populations since the type of soil, environmental conditions and level of anthropogenic impact influences the number and diversity of soil microbiota (Atlas and Bartha, 1986; Wenderoth and Reber 1999). Rousseaux et al. (2001) reported a large majority (84%) of GN bacteria isolated from soil contaminated with atrazine. On the other hand, Wenderoth and Reber (1999) isolated higher numbers of GP strains from soil contaminated with heavy metals. However, increasing concentration of contaminant resulted in decreasing of proportion of GP/GN.

About 45% of the total bacteria (GP and GN) isolated in the glucose-containing medium showed some level of diuron degradation, against 33% of the isolated in

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medium with diuron. Considering only the strains that degraded more than 10 mg L^{-1} in 120 h of cultivation, the GN group had a higher number of strains with the highest removal rates. Among the 10 strains with the highest diuron dissipation rates, 8 are Gram-negative indicating that the GN were more efficient for diuron dissipation despite the lower number in relation to the total bacteria in the sampled soil. These results corroborate data of Castillo et al. (2011) that showed that the GN *Azotobacter* sp. presented highest ability to degrade the organochlorine endosulfan among bacteria isolated from soil of coffee crops contaminated with that acaricide. The higher ability of GN bacteria to degrade atrazine was also reported by Rousseaux et al. (2001).

The highest diuron degradation rate was observed with *S. acidaminiphila* TD4.7 (4.2 mg L^{-1} of diuron in five days). This species has been shown to degrade xenobiotic compounds such as diuron (Batisson et al., 2007); polycyclic aromatic hydrocarbons (PAHs) and aliphatic hydrocarbons (Assih et al., 2002; Juhasz et al., 2000), acrylamide (Lakshmikandan et al., 2014), the insecticide acetamiprid (*N*-[(6-chloro-3-pyridyl)methyl]-*N'*-cyano-*N*-methyl-acetamidine) (Tang et al., 2012), the cyanobacterial toxin microcystin (Chen et al., 2010) and the herbicide butachlor (Dwivedi et al., 2010). The second most effective strain was *B. cereus* TD4.31, which degraded 3.4 mg L^{-1} of the diuron (68%) in five days. This bacterium has been described as able to degrade phenol (Saroja et al., 2000), benzene (Dou et al., 2010), decabromodiphenyl ether (Lu et al., 2013), petroleum hydrocarbons (Al-Saleh and Obuekwe, 2014), dimethyl sulfide (Liang et al., 2015) and pyrethroid insecticides (Liu et al., 2015). The strain *Delftia* sp. TD3.31, was the third most active on diuron, and belongs to a genus able to degrade the herbicide atrazine (Vargha et al., 2005), phenolic compounds (Jimenez et al., 2012), and 2-, 3- and 4-chloroaniline (Yan et al., 2011). Only one report associates the genus *Bacillus* with diuron degradation (Bazot and Lebeau, 2009).

The strain *O. intermedium* showed 33% removal of diuron. There is no information about the degradation of diuron by this bacterium. Species from this genus are able to degrade phenolic compounds (Chang et al., 2007; Yan et al., 2011), PAHs (Wu et al., 2009; Arulazhagan and Vasudevan 2011), polychlorinated biphenyl (PCBs) (Murínová et al., 2014) and aliphatic hydrocarbon (Dashti et al., 2015). The strain *A. faecalis* that degraded 35% of the diuron has been reported as able to degrade endosulfan (Kong et al., 2013), phenol (Jiang et al., 2007) and PAHs (Xiao et al., 2010). The *Achromobacter* sp. showing 35% degradation of diuron belongs to a previously reported diuron-degrading genus (Devers-Lamrani et al., 2014; Kaufman, 1977). Other reports are available about the biodegradation of sulfonamides (Reis et al., 2014) and biphenyl (Hong et al., 2009; Stanbrough et al., 2013). The members of genus *Micrococcus* were previously reported to degrade diuron (Sharma et al., 2010) and bacteria from this genus degrade 2-nitrotoluene (Mulla et al., 2013),

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nitrobenzene (Zhao et al., 2011), PAHs and aliphatic hydrocarbon (Dellagnezze et al., 2014).

No information is available in the literature about degradation of diuron by *Enterobacter* although species from this genus have been extensively studied for degradation of xenobiotic compounds such as acrylamide and polyacrylamide (Buranasilp and Charoempanich, 2011), PAHs (Festa et al., 2013), PCBs (Adebusoye et al., 2008), aliphatic hydrocarbon (Hua et al., 2010), trichlorobenzene (Adebusoye et al., 2007), and the herbicide glyphosate (Kryuchkova et al., 2014). Similarly, *P. aeruginosa* is the most studied bacterium able to degrade aliphatic and aromatic hydrocarbons (Al-Saleh and Akbar, 2015) besides PAHs (Zhang et al., 2011) and PCBs (Hatamian-Zarmi et al., 2009) but there are no reports about the degradation of diuron. Finally, no reported in biodegradation studies was found involving *Leucobacter* genera.

The majority of the strains isolated degraded diuron with the release of 3,4-DCA, confirming the common demethylation and amide cleavage steps. This pathway has been observed previously for the bacterial degradation of diuron (e.g. Tixier et al., 2002; Sharma et al., 2010). In general, 3,4-DCA is the major metabolite formed and its toxicity can affect cell growth. In the case of the bacteria isolated in this study, the concentrations of 3,4-DCA detected accounted for only a small fraction of the diuron degraded, suggesting that these strains could be degrading 3,4-DCA completely. The 8 strains chosen for detailed studies of degradation pathway (s) confirmed their ability to degrade 3,4-DCA more efficiently that they degraded diuron (12 to 95%), except for *S. acidaminiphila* TD4.7 which was able to degrade 80% of diuron and only 20% of the 3,4-DCA.

Analysis of the cultivation media of *P. aeruginosa* TD2.3, *S. acidaminiphina* TD4.7 and *B. cereus* TD4.31 showed the presence of 3,4-dichloroacetanilide (3,4-CAC), corresponding to the acetylation of the 3,4-DCA molecule. This compound has already been described as a part of the metabolic route of diuron degradation (Tixier et al., 2002; Ellegaard-Jensen et al., 2014; Yao et al., 2011). The possibility that this acetylation is a defense mechanism against the toxic effect of 3,4-DCA was considered. This acetylation is catalyzed by plasmidal acetyl transferase, an enzyme described for *Pseudomonas* and *Bacillus*. The primary function described for this enzyme is related to resistance to aminoglycosides antibiotics (Takenaka et al., 2006; Takenaka et al., 2007). As the plasmid genes are commonly transferred among the microbial populations in the environment, the involvement of these genes and the enzymes encoded by them in the biotransformation of xenobiotics in many strains in the environment could be feasible. The differential enzyme inhibition and activation in the soil in presence of diuron was demonstrated by Tejada et al., 2017.

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4-Chloroaniline (4-CA), resulting from dehalogenation of the 3-carbon of the aromatic ring of 3,4-DCA, was identified as a metabolite in culture media of *M. luteus* TG3.30, *A. faecalis* TG4.48 and *Achromobacter* sp. TD2.18. Dehalogenation is important for the degradation of persistent organochlorine contaminants and follows different routes degrading on the redox conditions. Under aerobic conditions, as in this experiment, dehalogenation of aromatic compounds without ring cleavage could proceed by different mechanisms as described previously (Janssen et al., 2001): 1). The cleavage of carbon-chlorine bounds by the 2,4,6-trichlorophenol-4-monooxygenase that incorporates two oxygen atoms accompanied by dehydrodehalogenation with a catechol as intermediate as described in *P. putida*. 2) A mechanism, described for pentachlorophenol metabolism in *Sphingomonas chlorophenolica,* involving a tetrachlorohydroquinone reductive dehalogenase (TCD) that catalyses the reductive displacement of a chlorine from a ring structure by a glutathione-dependent enzyme. 3) Dehalogenation involving a 4-chlorobenzoyl coenzyme A (CoA) dehalogenase, related to a 2-enoyl-CoA hydratase, with hydrolytic cleavage of the carbon–halogen bond and substitution of chlorine by an OH group from water with release of HCl. These reactions have been found in *Pseudomonas* sp CBS3, *Azotobacter* sp., *Sphingomonas chlorophenolica* ATCC 39723 *Burkholderia cepacia* AC1100 and *Nocardia* sp (Zaborina et al., 1998; Copley 1998; Yang et al., 1994; Klages and Lingens 1979). The fact that catechol was not detected in the culture media of the 3,4-DCA-degrading strains and its acidification during the cultivation may indicate a hydrolytic dechlorination of 3,4-DCA with release of HCl. The detection of the acetylated compounds 3,4-CAC and 4-CAC in cultures exposed to 3,4-DCA and 4-CA showed that bacteria were able to acetylate both chloroaniline compounds.

The results obtained in this study are in agreement with previous reports that degradation of diuron to 3,4-DCA follows a common route. However, the further aerobic degradation of the 3,4-DCA molecule can follow several routes. As reviewed by Giacomazzi and Cochet (2004), 3,4-dichloro-N-(3,4-dichlorophenyl) benzamide, 3,4-dichloroacetanilide, dichlorobenzene, 4-chloroaniline and dichlorocatechol can be metabolites of 3,4-DCA depending on the bacterial strains. The formation of 4-chloroacetanilide from 4-CA by inserting the acetyl group occurs in a few bacterial strain (Kaufman et al., 1973).

The growth of *O. intermedium* TG3.48, *P aeruginosa* TD 3.4 and *Achromobacter* sp TD 2.28 in media with aniline as the only carbon source, demonstrates the ability of these strains to open the aromatic ring. Since the ability to open the aromatic ring was not demonstrated for all strains, it is evident that interaction among different bacteria is essential to achieve the mineralization of diuron. Such interactions involve, in addition to the degradation steps, the attenuation of the toxic effects of the original compounds and their metabolites, through different pathways.

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Based on our results, we propose that the isolated bacteria show different abilities related to tolerance and degradation of diuron from the medium. The first group includes *P. aeruginosa* TD2.3, *S acidaminiphila* TD4.7, *B. cer*eus TD4.31 and *A. faecalis* TG 4.48, and acts on 3,4-DCA and 4-CA by alkylation and dealkylation, probably as adaptive mechanism for reducing the toxicity of the compound. The second group, including *M. luteus* and *Achromobacter* sp, follows the dehalogenation route directly to aniline, whose assimilation depends of the bacteria and their genetic characteristics (Fig. 6).

4. Conclusion

In conclusion, these results show that the role of microorganisms in the degradation of a xenobiotic in the environment is dependent on their individual metabolic capabilities as well as synergistic interactions with others member of microbial community. It is thus difficult to assess the importance of a particular strain when this is studied separately. The bacterial strains with the greatest potential to degrade diuron do not necessarily have the highest potential to degrade the metabolites

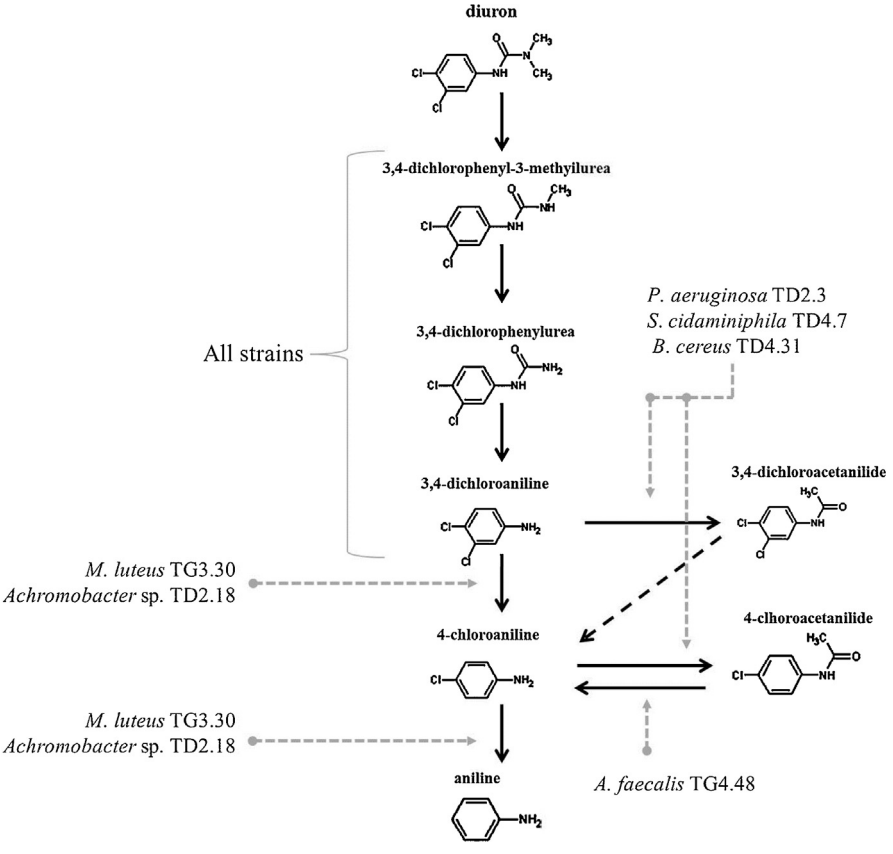

Fig. 6. Proposed pathway for the degradation of diuron by the bacteria isolated from soil of sugar cane crops.

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generated, reinforcing the idea that the mineralization of this compound is the result of microbial interactions. The presence of different metabolites indicates the involvement of enzymes such as demethylases, dehalogenases, acetylases and deacetylases in the complete degradation of diuron, which seem to be produced by different bacterial strains.

5. Materials and methods

5.1. Soil sampling

The soil samples were collected according to Egea et al. (2014), in a sugarcane crop area of Usina Açucareira Virgolino de Oliveira S/A (José Bonifácio, SP, Brazil, – Latitude 0610521 and Longitude 7657939) which had undergone consecutives applications of diuron in different herbicide formulations (such as Velpar-K® (diuron and hexazinona), Gamit® (clomazone) and Herburon® (diuron) for around seven years. The collection took place in two seasons of sugarcane growth (2012 and 2013) and the sample was taken after the herbicide application during the soil preparation and after harvest. Samples were taken at 10 cm depth, at three points belonging to the same row of the cultivation of sugarcane. The final sample was composed by mixing soils collected at the three points. Soil collected was placed in plastic containers in a Styrofoam box at room temperature for a maximum 3 h.

5.2. Isolation of the bacteria

The soil samples were ground until particles of about 1 mm and homogenized, under sterile conditions (Olsen and Bakken, 1987). Aliquots of 5 g were used for pre-culture in 500 mL Erlenmeyer flasks containing 100 mL of minimal medium (Dellamatrice and Monteiro, 2004) composed of (g L^{-1}) 6.0 of $NaNO_3$, 1.5 of KH_2PO_4, 0.5 of KCl, 0.5 of $MgSO_4{\cdot}7H_2O$, 0.001 of $FeSO_4$, 0.001 of $ZnSO_4$, at pH 6.8. The carbon source consisted of 500 mg L^{-1} glucose or 500 mg L^{-1} glucose plus 50 mg L^{-1} of diuron. The diuron was diluted in methanol, homogenized by two sonication pulses of 15 min before addition to the media. For each carbon source, five cultures were carried out under orbital shaking at 100 rpm, at 30 °C for 120 h and serial dilution of theses cultures were made with inoculation of aliquots of 100 μL of 10^{-2} to 10^{-6} dilutions in Petri plates containing the same media describe above. From Petri plates corresponding to dilutions of 10^{-4} and 10^{-6}, 10 colonies were isolated resulting in 100 colonies for each collect and a total of 400 colonies. All the isolated colonies were submitted to Gram tests and evaluation of their purity. The strains were maintained in 2% of glycerol solution, at −80 °C.

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5.3. Bacteria identification

The bacteria identification was done according Turnbull et al. (2001) with partial amplification and sequence of the *operon* ribosomic rRNA 16S. The primers 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGG-3′) were used to amplify 1300 pd (Marchesi et al., 1998). The sequences were compared to data from Genbank (http://www.ncbi.nem.nih.gov) and CBS (http://www.cbs.knaw.nl/index.htm).

5.4. Evaluation of diuron degradation

All the 400 isolated bacteria were cultivated in 50-mL Erlenmeyer flasks containing 10 mL of medium consisting of ($g.L^{-1}$) 0.05 of diuron, 5.0 of glucose, 6.0 of $(NH_4)_2SO_4$, 1.0 of KH_2PO_4, 2.0 of Na_2HPO_4, 0.5 of NaCl, 0.5 of Triton X–100. A volume of 100 μL from a micronutrient solution, consisting of ($g.L^{-1}$) 0.025 KI, 0.025 LiCl, 0.05 $MnSO_4$, 0.5 H_3BO_3, 0.05 $ZnSO_4$, 0.05$CoCl_2$, 0.025 $(NH_4)_6Mo_7O_{24}$, 0.05$CuSO_4$, 0.01 $FeSO_4$) and 10 μL of vitamin solution ($mg.L^{-1}$) 2.0 of biotin, 2.0 of folic acid, 5.0 of thiamin chloride, 5.0 nicotinic acid, 5.0 of calcium pantothenate, 10.0 of pyridoxine, 5.0 of lipoic acid and 0.1 of vitamin B12) were used. The pH was adjusted to 6.8.

To obtain the pre-inoculum, 500 μL of a bacteria suspension in 10 $g.L^{-1}$ of NaCl at OD_{600} = 0.3 were inoculated into 10 mL of the medium described above. The incubation was at 30 °C and 100 rpm, for 5 days. The weight of the flask containing medium and inoculum was determined at the beginning of the experiments and at the moment of sampling in order to control for volume reduction by evaporation. If decreasing volume occurred, sterile distilled water was used to adjust the volume.

After the incubation period, the content of the one flask was centrifuged at 6000 x g for 4 min and the supernatant was utilized to determine the remaining diuron concentration and the difference between cultivated medium and abiotic control (medium without inoculum) was included in the calculation. The metabolites released from diuron degradation were quantified in a similar way.

5.5. Diuron and derivatives determination

Before HPLC analysis, the samples were prepared by solid phase extraction (SPE) according to Gatidou et al. (2005). The sample treatment in polymeric cartridges (Oasis HLB, 60 mg) was done in according the manufacturer's instruction (Waters). 10 mL of the culture medium, centrifuged and filtered (0.22 μm filter), were applied to polymeric cartridges in a manifold (Supelco −12 samples) coupled to a vacuum pump (Marconi) at a flux of 6 $mL.min^{-1}$. The cartridges were

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conditioned with 3 mL of methanol and 3 mL of ultra-pure water and were eluted with 1 mL of methanol.

These samples were analyzed by high performance chromatography (HPLC) on a Perkin Elmer Model Flexar instrument. The injection volume was 10 μL using an Agilent Zorbax Eclipse Plus C18 (250 mm x 4,6 mm x 5 μm – PN: 959990–902) column and isocratic elution with water/acetonitrile (40/60 v/v) as mobile phase at a flow rate of 1.0 $mL.min^{-1}$ at 40 °C. The UV detector was set at 240 nm and run time was 9 min. Calibration curves were determined at concentrations from 0.5 to 50 mg L^{-1} for diuron and 0.05 to 10 mg L^{-1} for the metabolites. The correlation coefficients were greater than 0.999 for all curves.

Analysis by liquid chromatography coupled to mass spectrometry (LC-MS) on an Agilent 1220 Infinity LC-500 Ion Trap instrument. As mobile phase water was used with 0.2% of formic acid (A) and methanol with 0.2% of formic acid (B) with the following gradient: 20% B at 0 min, 90% B at 14 min, 90% B at 15 min, 20% B at 16 min, 20% at 18 min. The nebulizer gas pressure was 35 psi; drying gas pressure was 10 psi; drying gas temperature was 350 °C; needle voltage was 5000; and capillary voltage was 35.

The GC–MS analyzes were performed on a Perkin Elmer 680 gas chromatograph equipped with a Perkin Elmer – Clarus 600T mass selective detector. The analysis was carried out using an Elite 5-MS capillary column (Perkin Elmer – 30 × 0.25 mm, 1 μm) in splitless mode using helium as carrier at a flow rate of 1.0 $mL.min^{-1}$. The injected sample volume was 1 μL. The column temperature profile was programmed as follows: hold at 40 °C for 4 min; increase of 15 °C/min to 250 °C/ hold for 5 min; increase of 25 °C/min to 330 °C/hold for 10 min. The injector temperature was set at 270 °C. The interface temperature and ion source temperature were both set to 280 °C. Mass spectra were obtained using scan mode (mass range m/z 50 to 300) and SIR mode for monitoring the molecular ion and its fragments, with electron energy 70 eV, and using Turbo Mass software.

The analytical standards were purchased from Sigma Aldrich, LGC Standards GmbH and Da Vinci Laboratory Solutions. Ten diuron derivatives (3-(3,4-dichlorophenyl)-1-methylurea (DCPMU), 3-(3,4-dichlorophenyl)-urea (DCPU); 3,4-dichloroaniline (3,4-DCA); 4-chloroaniline (4-CA); 4-chloroacetanilide (4-CAC); aniline; 4-chlorocatechol; catechol; 1,2-dichlorobenzene (1,2-DCB); benzene) were used as standards for HPLC, LC-MS and GC-MS analyses.

Declarations

Author contribution statement

Tássia C. Egea: Performed the experiments.

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Maurício Boscolo, Janaina Rigonato, Diego A. Monteiro, Roberto Da Silva, Danilo Grünig, Humberto da Silva: Analyzed and interpreted the data.

Frans van der Wielen, Rick Helmus: Contributed reagents, materials, analysis tools or data.

John R. Parsons: Analyzed and interpreted the data; Wrote the paper.

Eleni Gomes: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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