Biodegradation (2005) 16: 275–282

3-Chloro-, 2,3- and 3,5-dichlorobenzoate co-metabolism in a 2-chlorobenzoate-degrading consortium: role of 3,5-dichlorobenzoate as antagonist of 2-chlorobenzoate degradation

Metabolism and co-metabolism of chlorobenzoates

Grazia Baggi[1,*], Silvana Bernasconi[2] & Maurizio Zangrossi[1]
[1]*Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi, Via Celoria 2, Milan 20133, Italy;* [2]*Dipartimento di Chimica Organica e Industriale, Università degli Studi, Milan, Italy (*author for correspondance: e-mail: grazia.baggi@fastwebnet.it)*

Accepted 14 July 2004

Key words: antagonism, co-substrate, halobenzoate dioxygenase, inhibition, microbial growth

Abstract

A study was made of the metabolic and co-metabolic intermediates of 2- and 3-chlorobenzoate, 2,3- and 3,5-dichlorobenzoate to elucidate the mechanism(s) involved in the negative effects observed on the growth of a chlorobenzoate-degrading microbial consortium in the presence of mixed chlorobenzoates. 2-Chloromuconate accumulated as the end-product in the cultural broths of the microbial consortium during growth on 2-chlorobenzoate; the same 2-chloromuconate was identified in the reaction mixtures of resting cells pregrown on 2-chlorobenzoate and exposed to 3-chloro- and 2,3-dichlorobenzoate, while in similar experiments 1,2-dihydroxy-3,5-dichloro-cyclohexa-3,5-dienoate was detected as dead-end product of 3,5-dichlorobenzoate co-metabolism. These results suggest an initial degradative attack by 2-chlorobenzoate induced dioxygenase(s). The role of 3,5-dichlorobenzoate as an antagonist of 2-chlorobenzoate degradation was also studied: in the presence of mixed 2-chloro- and 3,5-dichlorobenzoate, the 3,5-dichlorobenzoate preferential uptake by the resting cells of the chlorobenzoate-degrading consortium was observed. 2-Chlorobenzoate entered the cells only after the complete removal of the co-substrate. In growing cells experiments, the addition of 1,2-dihydroxy-3,5-dichloro-cyclohexa-3,5-dienoate, the 3,5-dichlorobenzoate co-metabolite, to 2-chlorobenzoate exerted the same antagonistic effect of the parent compound, inhibiting both the microbial growth and the degradative process. These data are discussed, allowing us to attribute the inhibitory effects observed to a substrate/co-substrate competition, though other additional causes may not be totally excluded.

Abbreviations: CB – chlorobenzoate; 2m.c. – 2-chlorobenzoate-degrading mixed culture; 2-CM – 2-chloromuconic acid; 3-CM – 3-chloromuconic acid; 3,5-diCB diol – 1,2-dihydroxy-3,5-dichloro-cyclohexa-3,5-dienoic acid

Introduction

Soils with a history of polychlorinated biphenyl (PCB) contamination have been found to contain chlorobenzoates (CBs) in isomer mixtures as the end-product of PCB congener cometabolism (Bedard 1990). Furthermore, to elucidate the bacterial catabolism of halogenated aromatics studies have been carried out on the degradative pathways of single CB isomers as model compounds (Baggi 2002; Haggblöm 1992). In recent years a different ecological approach has been adopted, and studies

are now being directed towards obtaining microbial cultures with a wide spectrum of degradation towards CB isomers, as such cultures could have potential in decontamination strategies useful for environments polluted with congener mixtures (Baggi & Zangrossi 2001; Fava et al. 1993; Hickey & Focht 1990; Miguez et al. 1990). Unfortunately, in some cases, the biodegradation properties of these microbial cultures can be quite limited by the CB mixtures often present at PCB polluted sites. The causes of these antagonistic effects, already reported for other pollutants of environmental concern such as BTX and PAHs (Alvarez & Vogel 1991; Baggi 2000; Bouchez et al. 1995; Stringfellow & Aitken 1995), have been ascribed to different phenomena like the toxicity of a mixture component or metabolic intermediate, competition for active enzyme sites involved in degradation and/or transportation to cells, interference in enzyme induction etc. (Alvarez & Vogel 1991; Baggi 2000; Baggi & Zangrossi 1999). In the particular case of CBs, the formation of 3-chlorocatechol(s) and the correlated *meta*-fission product(s), well known to be toxic to microorganisms (Bartels et al. 1984), has been proposed as a potential cause of inhibitory phenomena (Stratford et al. 1996). In earlier work we selected a versatile mixed culture (2m.c.) capable of using 2-chlorobenzoate (2-CB) as the sole C source and of degrading other mono- and diCBs when supplied singly; however this culture was inhibited in growing on the said 2-CB in the presence of CB co-substrates bearing one or two chloro-substituents in the *meta*-position, the only exception being 2,5-diCB that was used for growth by the said culture (Baggi & Zangrossi 2001). The present work investigated the metabolic and co-metabolic products of CB degradation to assess the possible involvement of these products in antagonistic effects, particular regard being given to 3,5-dichlorobenzoate (3,5-diCB) which was the most inhibitory compound.

Materials and methods

Cultural conditions and growing cell experiments

2M.c., previously selected on 2-CB as the sole C source and maintained in a liquid mineral medium with 0.1% of 2-CB (w/v), was used (Baggi & Zangrossi 2001). For growing cell experiments 2m.c. was cultured on 2-CB as the growth substrate, as described above, and 0.05% of 1,2-dihydroxy-3,5-dichloro-cyclohexa-3,5-dienoic acid (3,5-diCB diol) (w/v) was added, when required. Cell growth was monitored by measuring the optical density at 540 nm ($OD_{540\ nm}$).

Resting cell incubation experiments

For the resting cell experiments, 2m.c. cells grown on 2-CB were harvested by centrifugation (15′ at $12{,}000 \times g$) at the late exponential growth phase (30–48 h), washed twice with 0.02 M potassium phosphate buffer (pH 7), and re-suspended in the same fresh buffer to a final $OD_{540\ nm} = 1.2$ (about 2.5 mg dry weight ml^{-1}). The obtained cell suspension was diluted 1:2 with the same phosphate buffer and added with 0.05% of the tested CB(s) (w/v). After incubation for the appropriate time at 30 °C the suspension was ready for processing.

Extraction of metabolites

The cell-free culture fluids and the resting cell suspensions were centrifuged (15′ at $12{,}000 \times g$), acidified to pH 2 and extracted three times with ethyl acetate. The extracts were dehydrated by freezing and evaporated to dryness. The residues were identified by analytical procedures.

Analytical methods

The CB decrease and the appearance of intermediates in the growing and resting cell experiments were determined by HPLC; 2.5 ml of the cultural broth or reaction mixture were acidified with conc H_3PO_4 (5 μl) and added with CH_3OH (2.5 ml). The suspension was filtered (0.2 μm) and injected into a Jasco instrument (mod. LG 980-02 Ternary gradient Unit with UV detector λ_{max} 254 nm mod. UV-975 Intelligent UV/VIS) equipped with a Spherisorb ODS 2-04-0525 RP18 column (mobile phase CH_3OH/H_2O 58:42). The metabolites, evidenced by HPLC and extracted as described above, were identified by ^{1}H-NMR spectra recorded on a Ac200 Bruker (200 MHz) (solvent: DMSO-D6), and by mass spectra recorded on a VG 7070 EQ instrument (EI mode). The isolated 3,5-diCB diol was used as standard to determine the time course of 3,5-diCB co-metabolic transformation.

Chemicals

Organic and inorganic reagents of p.a. purity grade were from Aldrich (Milan, Italy).

Results

Degradation of 2-CB

In the growing cell experiments 2-CB degradation was followed until the complete disappearance of the growth substrate (0.1%, w/v). HPLC analyses of the culture revealed: at about 24 h there was the appearance of a new peak (Rt = 4.0′), that persisted through to the end of 48 h incubation; after 30–48 h incubation the peak attributable to 2-CB (Rt = 6.5′) completely disappeared. The acidic phase of the organic extracts was used to identify the new compound in the cultural broth; it was 2-chloromuconic acid (2-CM) and it represented about 10% (w/w) of the growth substrate. The structure of this metabolite was determined by mass spectrometry and ^{1}H-NMR. The molecular ion of 2-CM showed the presence of a chlorine atom ($M^+ + 2$ is approximately one third of M^+) and all the main peaks in the EI mass spectrum were in agreement with the proposed structure (Table 1). The ^{1}H-NMR spectrum showed the behaviour typical of muconic acids, the signals shifting upfield after adding D_2O and Na_2CO_3 (Jaroszewski et al. 1986) (Table2).

Co-metabolic degradation of 3-CB, 2,3-diCB and 3,5-diCB

The resting cells of 2m.c., pre-grown on 2-CB, were exposed to 3-CB, 2,3-diCB and 3,5-diCB (0.05%, w/v). Samples of the reaction mixtures were withdrawn at different incubation times and monitored for co-substrate disappearance and co-metabolite production.

3-CB

After 3 h incubation, HPLC analysis of the reaction mixture showed the complete disappearance of the peak ascribable to 3-CB (Rt = 8.7′), and the appearance of a major peak with the same retention time (Rt = 4.0′) as the product in the 2-CB cultures. ^{1}H-NMR spectra and mass-spectrometric analyses of the acidic phase of the organic extracts revealed 2-CM to be the major component. The co-metabolite that accumulated in the reaction mixture represented about 20% (w/w) of the original compound.

2,3-diCB

Within 27 h incubation the HPLC analyses of the reaction mixture showed the complete disappearance of the co-substrate (Rt = 8.6′), and the formation of a single co-metabolite, present almost stoichiometrically, with an Rt = 4.0′, identical to the compound identified in the 2-CB cultural broth and in the reaction mixture containing 3-CB. The product of the 2,3-diCB co-metabolism was con-

Table 1. Electron impact mass spectral data of the isolated metabolites

Metabolites	MS m/z (% relative intensity)
2-CM	178 (2%), 176 (6%), [M^+]; 161 (2%), 159 (6%) [$M^+ - OH$]; 141 (100%) [$M^+ - Cl$]; 133 (33%), 131 (100%) [$M^+ - OH - CO$]; 123 (30%) [$M^+ - H_2O - Cl$]; 115 (7%), 113 (18%) [$M^+ - OH - CO - H_2O$]; 95 (78%) [$M^+ - CO - HCl$]
3,5-diCB diol	228 (6%), 226 (3%), 224 (1%) [M^+]; 210 (2%), 208 (7%), 206 (10%) [$M^+ - H_2O$]; 192 (8%), 190 (20%), 188 (30%) [$M^+ - 2H_2O$]; 166 (10%), 164 (60%), 162 (100%) [$M^+ - H_2O - CO_2$]; 133 (25%), 115 (25%), 99 (57%)

Table 2. ^{1}H-nuclear magnetic resonance spectral data (200 MHz) of the isolated metabolites

Metabolites	^{1}H-NMR (solvent) (multiplicity, number of protons, attibution)
2-CM	(DMSO-D6), δ: 8.10 (d, 1H, C**H**—CCl), 7.45 (t, 1H, CH=C**H**—CH) , 6.0 (d, 1H, C**H**COO)
	(DMSO-D6 + D_2O + Na_2CO_3), δ: 7.50 (d, 1H, C**H**—CCl), 6.88 (t, 1H, CH=C**H**—CH), 5.80 (d, 1H, C**H**COO)
3,5-diCB diol	(DMSO-D6), δ: 6.15 (bs, 1H, C**H**=C), 5.85 (bs, 3H, C**H**=C + 2 —O**H**), 4.45 (bs, 1H, C**H**—O)

firmed to be 2-CM by ^{1}H-NMR and mass-spectrometry of the acidic phase of the organic extract.

3,5-diCB

At 24–30 h incubation the HPLC analyses of the reaction mixture in the presence of 3,5-diCB showed the complete disappearance of the co-substrate (Rt = 17.5′) and the appearance of a co-metabolite having Rt = 4.4′, that was not transformed further and represented about 50% (w/w) of the co-substrate supplied. On the basis of the data obtained from mass-spectrometry and ^{1}H-NMR (Tables 1 and 2), the structure of this co-metabolite was attributed to 1,2-dihydroxy-3,5-dichloro-cyclohexa-3,5-dienoic acid (3,5-diCB diol). In the mass spectrum the molecular ion showed two chlorine atoms, corresponding to 3,5-diCB with two hydroxyl groups, the main peak corresponding to 3,5-dichlorophenol (m/z 166, 164, 162). The ^{1}H-NMR spectrum confirmed the proposed structure. In the Table 3 the time course of the 3,5-diCB cometabolic transformation was shown.

Effect of 3,5-diCB on 2-CB degradation by the mixed culture

Several Authors have shown 3,5-diCB to have a strong inhibitory effect on the growth of different microorganisms in pure culture when the dichloro-aromatic was supplied in combination with another CB as growth substrate (Baggi & Zangrossi 1995; Hernandez et al. 1991; Miguez et al. 1990; Stratford et al. 1996). In a previous work we observed a similar effect on the growth of 2m.c. when supplied with 2-CB in combination with the same 3,5-diCB (Baggi & Zangrossi 2001). This prompted us to investigate the cause of the 2m.c. behaviour in the presence of mixed CBs by conducting experiments with growing and resting cells.

Table 3. Time course of the co-metabolic transformation of 3,5-diCB[a]

	3,5-diCB (%)	3,5-diCB diol (%)
T_0	0.50	0.00
4 h	0.39	0.07
6 h	0.33	0.10
16 h	0.12	0.20
24 h	0.00	0.28

[a] Values obtained from resting cell experiments. The experimental conditions are detailed in materials and methods.

Growth experiments

To study the effect of the 3,5-diCB diol, that we had obtained from 3,5-diCB co-metabolism, on the 2m.c. growth, a culture supplied with 0.05% (w/v) of it in mixture with 2-CB (0.1%, w/v) was performed as described in Materials and methods. The $OD_{540\ nm}$ of this culture was measured until 4 days of incubation; the control for comparison contained only 2-CB as the growth substrate. The results showed that also in the presence of the 3,5-diCB diol the growth of 2m.c. on 2-CB was completely inhibited, in agreement with the results of previous experiments conducted with 3,5-diCB in combination with 2-CB (Baggi & Zangrossi 2001).

Resting cell experiments

To follow the uptake of 2-CB and 3,5-diCB in combination supplied, (both at 0.05% w/v) to the 2m.c. resting cells pre-grown on 2-CB, we monitored the CB concentration in supernatant samples by HPLC analyses at different incubation times. For control purposes, the changes in concentration of 2-CB and 3,5-diCB, both supplied singly at 0.05% (w/v), were also determined. The results showed that the two compounds singly supplied freely entered into the cells, though at different rates: in fact 2-CB and 3,5-diCB totally disappeared from the supernatants within about 6–24 h of incubation. On the contrary, when the two compounds were present in mixture, 3,5-diCB concentration time course was the same of the control, while 2-CB concentration remained unchanged until 3,5-diCB had totally disappeared from the supernatant. Then, 2-CB started to be slowly taken up by the cells, totally disappearing from the supernatant within 44 h incubation (Table 4).

Discussion

In the present work we identified 2-CM and 3,5-diCB diol as the end-products of the metabolic and co-metabolic degradation of 2- and 3-CB, 2,3- and 3,5-diCB in a highly versatile mixed culture that we had selected in earlier work (Baggi & Zangrossi 2001). The detection of 2-CM in 2-CB cultural

Table 4. Uptake course[a] of 2-CB and 3,5-diCB, singly and in mixture supplied

	2-CB (%)	3.5-diCB (%)	2-CB (%) + 3.5-diCB (%)
T_0	0.50	0.50	0.50 + 0.50
4 h	0.17	0.39	0.50 + 0.36
6 h	0.11	0.33	0.50 + 0.30
16 h	0.04	0.12	0.50 + 0.05
24 h	0.00	0.00	0.49 + 0.00
44 h	n.d.	n.d.	0.00 + 0.00

n.d. = not determined.
[a] Expressed as CB concentration in resting cell supernatants at different incubation times. The experimental conditions are detailed in Materials and methods.

broths is in line with the involvement of a benzoate 1,6-dioxygenase, induced by 2-CB, to give the corresponding diol transformed into 3-chlorocatechol. In fact it is the *ortho*-cleavage of 3-chlorocatechol that gives the 2-CM that accumulates in the culture. As 2-CB is the sole growth substrate and 2-CM represents only a fraction of the original compound, it is most probable that in our consortium the 2-CB degradation proceeded through other metabolic pathways like dioxygenation at the 1,2-position, to give a diol that is spontaneously decarboxylated and dehalogenated to catechol by the action of the same, or another, dioxygenase induced by 2-CB in 2m.c.. In such a case the catechol would have reacted further to supply energy for cell growth (Figure 1a). Although there was no evidence of catechol formation, probably because it is transient, this hypothesis is in line with the literature on 2-CB degradation, that indicates the prevailing route to be the formation of muconic acid, brought about by the initial attack of a 1,2-dioxygenase, induced by *ortho*-substituted haloaromatics (Engesser & Schulte 1989; Hartmann et al. 1989; Hernandez et al. 1991; Kozlovski & Kunc 1995; Romanov & Hausinger 1994). Moreover, our hypothesis of the coexistence of two pathways, *via* catechol and 3-chlorocatechol, has already been demonstrated in two *Pseudomonas* strains and in *Burkholderia,* able to grow on 2-halobenzoates (Francisco et al. 2001; Sylvestre et al. 1988; Vora et al. 1988). Similar CB dioxygenases exhibiting different substrate specificity towards CBs, with particular regard to the *ortho*-substituted analogues, were characterised some time ago (Fava et al. 1996; Fetzner et al. 1992). Recently, even the genes for catechol and the 3-chlorocatechol *ortho*-catabolic pathways were identified and sequenced in different strains (Corbella et al. 2001; Francisco et al. 2001; Haak et al. 1995; Suzuki et al. 2001; Tsoi et al. 1999).

The accumulation of 2-CM, also from 3-CB co-metabolism, demonstrated that in our 2m.c. this co-substrate was attacked at the 1,2-position, giving the same end-product as in the 2-CB cultures. However, because of the complete disappearance of the co-substrate and the non stoichiometric yield of the co-metabolite, it cannot be excluded that 3-CB might also be co-metabolised by another initial attack, i.e. dioxygenation at the 1,6-position to give 4-chlorocatechol that is never detected as it is degraded completely (Figure 1a). In line with our hypothesis are the findings of Pieper et al. (1993) who obtained substantial amounts of 2-CM through a minor 3-CB degradation pathway in *Alcaligenes eutrophus* JMP 134; the major degradation pathway proceeded through the formation of 4-chlorocatechol, derived from an attack at the 1,6-position of 3-CB. 3-CM that resulted from the *ortho*-fission of 4-chlorocatechol, was further and efficiently degraded, being a good substrate for chloromuconate cycloisomerase, while 2-CM accumulated because of its poor affinity for the said cycloisomerase.

With regard to 2,3-diCB, the literature reveals that some strains can grow on it (Hernandez et al. 1991; Hickey & Focht 1990; Miguez et al. 1990), but evidence of its catabolic pathway has only been obtained with *P. aeruginosa* JB2. Both 2,3-dichlorophenol and 4-chlorocatechol were detected in resting cell experiments, and it was postulated that they were due to dioxygenation at the 1,6-position of the dichloroaromatic (Hickey & Focht 1990). The former compound was probably produced by the acid-catalysed dehydration of the corresponding dichlorinated diol, while, in a less convincing hypothesis, 4-chlorocatechol was postulated as being derived from the same diol that, to the best of our knowledge, does not dehalogenate spontaneously. In our case, the almost stoichiometric formation of 2-CM can be explained only by hypothesising a dioxygenative attack at the 1,2-position of 2,3-diCB, followed by the spontaneous detachment of the chlorine in C2; this determines, yet again, the convergence of the 2,3-diCB pathway with the pathways of 2-CB and 3-CB (Figure 1b). The 3,5-diCB co-metabolic degradation gave 3,5-diCB diol as end-product and the

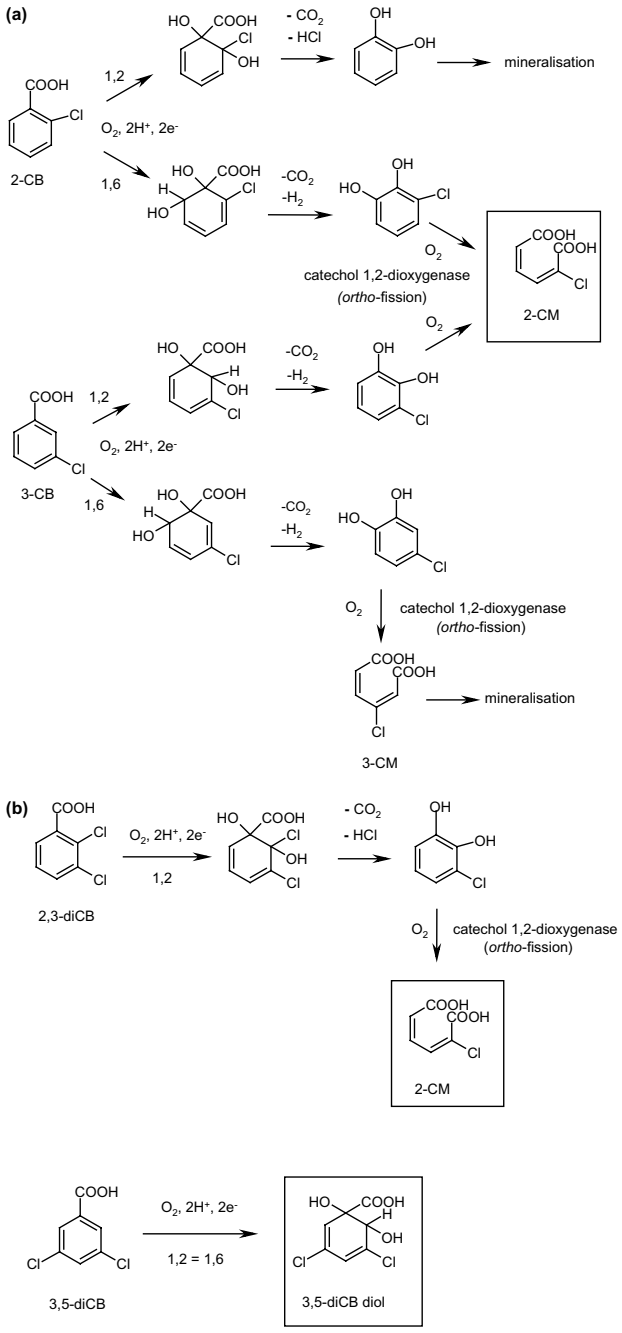

Figure 1. Attack of benzoate dioxygenase(s) induced by 2-chlorobenzoate on mono- (a) and dichlorobenzoates (b). The compounds in the squares have been isolated and identified.

time course of such transformation is shown in Table 3. This result further confirms the involvement of a 2-CB induced dioxygenase that, in this case, acts quite indifferently at both the 1,2- and the 1,6-position because of the diCB symmetry (Figure 1b). This is in agreement with the data obtained by Hernandez et al. (1991), who identified 3,5- and 2,4-dichlorophenol, derived from decarboxylation and dehydratation of the said diol. The accumulation of the 3,5-diCB diol was

explained by the lack of induction of the functional dihydrodiol dehydrogenase needed for the subsequent degradation of the diol that cannot dehalogenate spontaneously. Obviously, as 3,5-diCB was not the growth substrate in either of the cases, the induction of the necessary dehydrogenase did not occur. In conclusion, these results highlight the ability of our 2-CB-degrading mixed culture to co-metabolically transform, to different degrees, 3-CB, 2,3- and 3,5-diCB when supplied singly to the resting cells, such transformation occurring through an initial attack by dioxygenase(s) induced by 2-CB alone. Interestingly, the same compounds inhibited the growth of the culture when supplied together with 2-CB, as has already been reported (Baggi & Zangrossi 2001). In fact we and other Authors have reported the occurrence of antagonistic effects on microbial growth when CB mixtures were supplied to CB-degrading microorganisms (Baggi & Zangrossi 1995, 1999; Miguez et al. 1990; Stratford et al. 1996). In all cases 3,5-diCB was shown to be an inhibitory compound when present in combination with another CB as growth substrate. The causes for this were not always elucidated, or were tentatively attributed to the toxicity of some metabolites like 3-chlorocatechol(s) and its *meta*-fission product(s), as suggested by Stratford et al. (1996), who, however, did not detect such intermediates in the cultures. In another case, interference in degradative enzyme induction has been postulated as being involved in the failure of *Alcaligenes denitrificans* CB strain to grow on 4-CB in the co-presence of 3,5-, 3,4- and 2,3-diCB. The reason put forward is that the O_2 uptake of the resting cells, already equipped with catabolic enzymes, is not inhibited in the presence of mixed CBs (Baggi & Zangrossi 1999). On the other hand, the inhibition exerted by 3-CB on the growth of *Pseudomonas* sp. strain B 13 on benzoic acid was attributed to competition for the active sites of the carriers, or degradative enzymes involved in the uptake or metabolism of the growth substrate, while similar inhibitory phenomena among CBs observed in *Alcaligenes denitrificans* BRI 3010 and BRI 6011, were ascribed to the transport systems involved (Miguez et al. 1995). In the case of the 3,5-diCB inhibition found for our microbial consortium, the cause cannot be attributed to the formation of metabolites like 3-chlorocatechol and its *meta*-fission product: in fact the isolation of 2-CM as the end-product of 2-CB degradation and 3-CB and 2,3-diCB co-metabolism, clearly demonstrates the *ortho*-fission of the chlorine-substituted aromatic nucleus, while the co-metabolism of 3,5-diCB proceeds only to the formation of the corresponding diol without the opening of the ring. The inhibition of microbial growth exerted not only by 3,5-diCB, but also by 3,5-diCB diol, the latter up to date never reported, can, presumably, be attributed to the presence of the two chloro-substituents at 3 and 5 position on both the parent compound and its co-metabolic product. The inability of the 2m.c. resting cells to take up 2-CB when 3,5-diCB is present, shown by the unchanged 2-CB concentration outside the cells exposed to mixed 2-CB and 3,5-diCB, suggests that this behaviour may be attributed to 2-CB and 3,5-diCB competition for the carriers, or for the degradative enzymes involved in CB degradation. This hypothesis is supported by the fact that when 3,5-diCB had totally disappeared from the reaction mixture, 2-CB began to be taken up by the cells (Table 4). A similar mechanism was reported by Miguez et al. (1995) for the above cited antagonism between 3-CB and benzoic acid in *Pseudomonas* sp. strain B13. Work in this direction is in progress to verify the role other CBs like 3-CB, 2,3- and 3,4-diCB could have in inhibiting 2m.c. growth on 2-CB. Such inhibitory phenomena that limit the degradation potential of microorganisms, could explain the failure of some bioaugmentation strategies when isomer or analogue mixtures are present.

Acknowledgements

Partial financial support by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MIUR), COFIN 2002–2003 is gratefully acknowledged.

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