The Degradation of 2-Chlorophenol in an Upflow Anaerobic Sludge Blanket (UASB) Reactor

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1 INTRODUCTION

Chlorophenols are widespread toxic compounds that are included in the U.S. Environmental Protection Agency list of priority pollutants. They are used in industry primarily as biocides as well as preservatives for wood, glue, paint, vegetable fibers and leather (Muller and Caillard, 1986). Chlorophenols may also be formed in the chlorination of surface waters and wastewasters. Additionally, chlorophenols have been found to be present in waste waster and sludges, sediments, ground water (due to leaching from contaminated soils), surface water (due to surface runoff or direct industrial waste discharges) and rainfall (cited in Krumme and Boyd, 1988). The toxicity of chlorinated phenols tends to increase with their degree of chlorination and because few microorganisms can decompose them, the more highly chlorinated phenols tend to accumulate in the environment.

Natural removal of chlorophenols from the environment can be achieved by photodecomposition and biodegradation. The biodegradation of chlorophenols has been studied in both, aerobic and anaerobic systems. Under anaerobic conditions chlorine substituents can be removed from the aromatic ring by reductive dechlorination. In this process chlorines are replaced by hydrogen, resulting in less toxic and less recalcitrant compounds. Anaerobic processes are reported to be suitable for the dechlorination of low to highly-chlorinated phenolic compounds while aerobic systems have a tendency to be more suitable for biodegrading the less-halogenated phenolic compounds.

The degradation of aromatic compounds

A large variety of aromatic substances participate in life processes and form an important part of the natural carbon cycle. In nature aromatic compounds are derivatives of the secondary metabolism of plants, biological and chemical cleavage of lignin, and bioconversion of aromatic amino acids (cited in Knoll & Winter, 1989). Furthermore, many aromatic compounds are produced by the chemical industry for utilization as pesticides, insecticides, herbicides, detergents, solvents, wood preservatives, etc. Some xenobiotic benzenoid structures are relatively recalcitrant, requiring the competence of the microbial world for their dissimilation.

Under aerobic conditions aromatic substrates are metabolized by a variety of bacteria, with ring fission accomplished by mono- and dioxygenases. Molecular oxygen is essential for these enzymes to function since it is incorporated into the reaction products. Aerobic degradation pathways and elimination reactions for substituents have been reviewed somewhere else (e.g. Evans, 1969; Dagley, 1971; Wallnöfer and Engelhardt, 1984; Schwien et al., 1988).

As early as 1934 the degradation of aromatics during methane fermentation was reported by Tarvin and Buswell (1934). All of the ring carbons of the aromatic substrate were accounted for as CO_2, CH_4 and microbial cells. This pioneering quantitative study of the methanogenesis of organic compounds established the stoichiometry of the reaction as follows:

$$C_nH_aO_b + (n - \frac{a}{4} - \frac{b}{2})\, H_2O \longrightarrow (\frac{n}{2} + \frac{a}{8} + \frac{b}{4})\, CO_2 + (\frac{n}{2} + \frac{a}{8} - \frac{b}{4})\, CH_4 \quad .....(1)$$

The metabolic fate of organic compounds and their mineralization to CO_2 (and CH_4) depends on the availability of light or of inorganic electron acceptors such as NO_3^-, SO_4^{-2}, or CO_2. Since 1952, a great number of publications have appeared on the breakdown of both, aromatic and other cyclic compounds under anaerobic conditions. Metabolism of the aromatic ring in the absence of molecular oxygen is now known to proceed in five different ways (Table 1.1).

(a) Anaerobic photometabolism : One of the products of the light reaction was a strong oxidant ($^{\bullet}OH$) which, in plants, was converted into molecular oxygen; in bacteria this proposedly light-induced "bound oxygen'' was used to oxidize substrates. Several species of the purple nonsulfur Rhodospirillaceae family can grow anaerobically in the light at the expense of simple aromatic compounds as sole carbon source.

(b) Under nitrate-reducing condition : Nitrate-reducing bacteria couple the oxidation of organic compounds with water to the exergonic reduction of nitrate via nitrite to N_2. Energy is derived mainly from electron transport phosphorylation during nitrate respiration and cell carbon is derived from breakdown products of the organic compound.

(c) Anaerobic dissimilation of aromatic compounds through sulfate respiration : Sulfate-reducing bacteria couple the oxidation of organic compounds with water to the exergonic reduction of sulfate via sulfite to sulfide. Energy and cell carbon are produced in the same principle manner as by nitrate-reducing bacteria. Sulfate reducers are mainly responsible for degradation of organic matter in anaerobic marine environments.

(d) The anaerobic fermentation of many polyphenolic substances: Microorganisms derive their energy from substrate-level phosphorylation while organic compounds serve as electron donors and acceptors. Patel et al. (1981) and Tsai & Jones (1975) were the first to isolate from the rumen species of Coprococcus and Streptococcus that fermented phloroglucinol via dihydrophloroglucinol to acetate.

(e) The methanogenic fermentation of naturally occuring soluble aromatic compounds by undefined consortia of bacteria cooperting to form a food chain : Balba and Evans (1980) used a methanogenic consortium to investigate catechol degradation via transformation to diol followed by dehydroxylation to phenol prior to reduction of the ring.

Biodegradation of haloaromatic compounds

The concern about numerous aryl halide pollutants came up during the 1970s. Aryl halides include pesticides, solvents, heat transfer fluids, and waste products from many

Table 1.1 Anaerobic metabolism of aromatic compounds (from Evans and Fuchs, 1988)

Energy-yielding process	Organism	Substrates
Photosynthetic phosphorylation	*Rhodopseudomonas palustris* *Rhodopseudomonas gelatinosa*	Benzoate *m,p*-Hydoxybenzoate
Denitrification $NO_3^- + 2H^+ + 4H_2 \longrightarrow NH^+_4 + 3H_2O$ $\Delta G'0 = -600$ kJ	*Pseudomonas* PN-1 *(Alcaligenes xylosoxidan)* *Moraxella* sp. *(Parococcus denitrificans)* *Bacillus* sp. *Pseudomonas*	Benzoate Hydoxybenzoate Protocatechuate *o,m,p*-Phthalate 2-Aminobenzoate Phenol o,m,p-Cresol
Sulfate reduction $SO_4^{-2} + 2H^+ + 4H_2 \longrightarrow H_2S + 4H_2O$ $\Delta G'0 = -152$ kJ	*Desulfovibrio* sp. *Desulfococcus* *Desulfonema* *Desulfosarcina*	Benzoate Hydroxybenzoate Phenylacetate, hippurate Phenol Indol
Fermentation	*Coprococcus* sp. *Streptococcus* *Pelobacter acidigallici* *Eubacterium oxidoreducens*	Phloroglucinol Resorcinol/acids Gallate, pyrogallol Polyphenols
Methanogenic fermentation $HCO^-_3 + H^+ + 4H_2 \longrightarrow CH_4 + 3H_2O$	Microbial consortia : fermentative bacteria + acetogenic and methanogenic bacteria	Lignin (cornstalks) Benzoate Tyrosine Vanillate phenylpropionate Phenylacetate, benzoate

$\Delta G'0 = -135$ kJ	Phenol , Catechol Hydroquinone Syringate Phenylalanine Chlorophenols Chlorobezoate Benzene, toluene Nitrophenols Chlorophenoxyacetate

industrial processes. These compounds include halogenated anilines, benzenes, biphenyls, phenoxyacetates and phenols. Among them, chlorinated aromatic compounds are major environmental pollutants because they are often released in substantial quantities, are toxic and resistant to degradation, and may accumulate in sediments and biota. The biological recalcitrance of halogenated compounds is related to the number, type, and position of the halogen substituents.

The carbon-halogen bond is regarded as increasingly recalcitrant with increasing electronegativity of the substituent. Halogenated substances with one or few substituents are thougt to be more readily degradable than the corresponding polyhalogenated compounds. The carbon-halogen bond can be cleaved either by enzymatic dehalogenation (catalyzed by specific enzymes) or by spontaneous chemical dehalogenation of unstable intermediates. Concerning the enzymatic cleavage of the carbon-halogen bond, seven mechanisms of dehalogenation are known so far :

1. Reductive dehalogenation : This reaction is a two-electron transfer reaction which involves the release of the halogen as a halogenide ion and its replacement by hydrogen.

2. Oxygenolytic dehalogenation : These reactions are catalyzed by mono-oxygenases (or dioxygenases), which incorporate one (or two) atoms of molecular oxygen into the substrate.

3. Hydrolytic dehalogenation : In the course of these reactions, catalyzed by halido-hydrolases, the halogen substituent is replaced in a nucleophilic substitution reaction by a hydroxyl group which is derived from water.

4. Thiolytic dehalogenation : In dichloromethane-utilizing bacteria, a dehalogenating glutathione S-transferase catalyzes the formation of a S-chloromethyl glutathione conjugate, with a concomitant dechlorination taking place.

5. Intramolecular substitution : Intramolecular nucleophilic displacement yielding epoxides is a mechanism involved in the dehalogenation of vicinal haloalcohols.

6. Dehydrodehalogenation : In dehydrodehalogenation, HX is eliminated from the molecule, leading to the formation of a double bond.

7. Hydratation : A hydratase-catalyzed addition of a water molecule to an unsaturated bond can yield dehalogenation of vinylic compounds e.g. 3-chloroacrylic acid, by chemical decomposition of an unstable intermediate.

Anaerobic dehalogenation reactions are of particular environmental interest. Such reactions have been intensively investigated with aliphatic and alicyclic pollutants. Aryl halogens can be released from a molecule only by reduction or hydrolytic dehalogenation reactions. Halide removal by hydroxylation is more frequently reported for heterocyclic aromatic compounds (Kuhn & Suflita, 1989) whereas reductive dehalogenation is more commonly associated with homocyclic aromatic substances (Young, 1984; Neilson et al., 1987; Kuhn & Suflita, 1989).

Reductive dehalogenation of halogenated aromatic compounds

Reductive dehalogenation is an important means of biodegradation of numerous compounds, including organochlorine pesticides, alkyl solvents, and aryl halides. This reaction is the only known biodegradation mechanism for certain significant pollutants such as highly chlorinated polychlorinated biphenyls (PCBs), hexachlorobenzenes (HCB), tetrachloroethene (PCE), and pentachlorophenol (PCP). Generally, reductive dehalogenation reactions are favoured under highly reducing methanogenic conditions (Gibson & Suflita, 1986) and often are found as the initial step in anaerobic biodegradation of most haloaromatic compounds.

Reductive dehalogenation involves the removal of a halogen substituent from a molecule with concurrent addition of electrons to the molecules. Concerning the mechanism of reductive dehalogenation, a reduced organic substrate or H_2 might be the source of both the reducing power and the protons. On the other hand, dehalogenation might occur in a two-step reduction by an electron donor (reduced organic substrate) and with proton abstraction from the solvent (Fetzner & Lingens, 1994). An example of the latter was the study of Nies and Vogel (1991). They showed that the source of the hydrogen added to the aromatic ring in reductive dehalogenation of 2,3,4,5,6-pentachloro-biphenyl is a proton from water.

The first lines of evidence for anaerobic reductive dehalogenation of halogenated aromatics were presented by Horowitz et al. (1982) and Sulflita et al. (1982). They found that an anaerobic microbial consortium isolated from sewage sludge could degrade a number of *meta*-substituted chlorinated benzoates. The most interesting degradative reaction was the loss of chloride without the alteration of the aromatic ring. When all the chlorine atoms were successively removed, ring fission led to methane and CO_2. Dolfing & Tiedje (1986)

established a defined 3-chlorobenzoate-degrading consortium consisting of a key organism, i.e. the dechlorinating organism (DCB-1), the benzoate degrader (BZ-1), and the lithotrophic methanogen (*Methanospirillum* strain PM-1). The chlorine released from the aromatic ring was recovered in stoichiometric amounts as chloride ion. The reducing power required for reductive dechlorination was obtained from the hydrogen produced in the acetogenic oxidation of benzoate. One third of this hydrogen was consumed via the reductive dechlorination, while two thirds were left for the methanogen

Reductive dechlorination has also been shown for 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), chlorophenols, and 1,2,4-trichlorobenzene (1,2,4-TCB). Even pentachlorophenol (PCP) was completely dechlorinated by a mixture of the 2-CP-, 3-CP-, and 4-CP-acclimated sludges (Mikesell & Boyd, 1986). The proposed PCP degradation pathway, based on the sequential appearence and disappearence of 3,4,5-TCP, 3,5-DCP, and 3-CP, appeared to result from the relatively higher rate of PCP dechlorination by the 2-CP-acclimated sludge. This sludge rapidly removed chlorine from positions 2 and 6 of PCP to give 3,4,5-TCP.

Hydrolytic dehalogenation of halogenated aromatic compounds

The first evidence implicating replacement of chlorine from the aromatic ring through a hydroxyl group were presented by Johnston et al. (1972). They isolated a *Pseudomonas* species capable of utilizing 3-chlorobenzoate as the sole carbon source. During growth with 3-chlorobenzoate, 3-hydroxy- and 2,5-dihydroxybenzoate were excreted into the culture medium. In 1975 Chapman isolated *Micrococcus* spp. able to grow with 4-chlorobenzoate. Degradation apparently proceeded via 4-hydroxybenzoate and protocatechuate.

The mechanism of the dehalogenation process has been clarified by labeling experiments using $^{18}O_2$ and $H_2{}^{18}O$ (Marks et al., 1984; Müller et al.,1984). The data indicated that the dechl orination reaction utilized water as the hydroxyl donor and not molecular oxygen. The results showed that the enzymatic conversion of 4-chlorobenzoate to 4-hydroxybenzoate proceeds via a hydrolytic cleavage of the carbon-chlorine bond.

Anaerobic biodegradation of chlorophenols

Both monochlorophenols and polychlorinated phenols can be degraded anaerobically but the reactive reaction has been shown to be more effective for dechlorination of compounds with multiple chlorine substitutions. Mineralization of chlorophenols in methanogenic environments often starts with reductive dechlorination to phenol and ends with formation of methane and carbon dioxide. Degradation is dependent upon

characteristics of the parent compound, the microbial consortium, and environmental factors. Reductive dechlorination of chlorophenols has been observed for unacclimated and acclimated anaerobic sewage sludges, sediments, soils amended with sewage sludge, and aquifers.

A number of studies reported reductive dechlorination and mineralization of mono-chlorophenols (MCPs) by mixed anaerobic cultures. Boyd & Shelton (1984) investigated the anaerobic degradation of mono- and dichlorophenol isomers by fresh sludge and by sludge acclimated to either 2-, 3-, or 4-chlorophenol. In unacclimated sludge, each of the MCP isomers was degraded. The rates of disappearence were in the order : *ortho* > *meta* > *para*. With sludge acclimated to the chlorophenols, the lag time preceding dechlorination decreased, rates of dechlorination increased, and distinct patterns of acclimation were observed. Sludges acclimated to 2-CP degraded 4-CP and 2,4-DCP but not 3-CP and those acclimated to 3-CP were incapable of degrading 2-CP, 2,3- and 2,5-DCP. The sludge acclimated to 4-CP could degrade all three MCP isomers and 2,4- and 3,4-DCP. Krumme and Boyd (1988) showed that an anaerobic upflow bioreactor, supplied with chlorinated phenols as the sole carbon and energy source, could reductively dechlorinate and partially mineralize monochlorophenols. Approximately 40 % of the added chlorophenols were mineralized to CH_4 and CO_2. However, this system was limited in its ability to degrade higher-chlorinated phenols. Dietrich and Winter (1990) reported complete degradation of 2-CP by an enriched anaerobic mixed culture from sewage sludge. Degradation rates of 2-CP of up to 0.18 g/l, day were observed in suspended cultures without biomass retention and of 0.375 g/l, d in cultures immobilized on Liapor clay beads. Sharak Genthner and her coworkers (1989) had compared anaerobic degradation of monochlorophenols and monochlorobenzoates in a variety of aquatic sediments under four enrichment conditions. They found that degradation of chloroaromatic compounds was observed most often in methanogenic enrichments and in enrichments amended with 1 mM bromoethane sulfonic acid (BESA) and least often in enrichments with added nitrate or sulfate. In contrast, the experiments from Haggblom and Young (1990 & 1995) showed that the chlorophenols were mineralized under sulfidogenic conditions and substrate oxidation was coupled to sulfate reduction, since substrate utilization was dependent on sulfidogenesis and chlorophenol loss did not proceed in the absence of sulfate.

Previous studies on the success of the anaerobic degradation of polychlorinated phenols using laboratory-scale reactors have been very limited. Mohn & Kennedy (1992) reported the limited ability of sludge granules from upflow anaerobic sludge blanket (UASB) reactors in degrading two trichlorophenols (TCP) and one dichlorophenol (DCP) in batch incubations. The sludge granules were capable only of partial degradation of the tested chlorophenols and did not appear to be capable of mineralization of chlorophenols with *meta*- or *para*-

chlorine substituents. Madsen and Aamand (1992) found evidence of a 2,4,6-TCP dechlorinating bacterial population that regained dechlorinating activity after the culture was heated to 80°C for an hour, suggesting that the dechlorinating bacteria were presumably spore-forming anaerobes. This culture was capable of reductively dechlorinating 2,4,6-TCP via 2,4-DCP to 4-CP, which was a persistent degradation product. Zhang and Wiegel (1990) reported 4-CP to be present for more than 50 days before being degraded in an acclimated fresh water sediment that dechlorinated 2,4-DCP. Flora et al.(1994) reported how toxicity due to the intermediate product (4-CP) is controlled through carbon displacement of the anaerobic fluidized-bed granular activated carbon (GAC) reactor treating a simulated high-strength industrial wastewater containing inhibitory concentrations of chlorophenols. Krumme and Boyd (1988) reported the degradation of 3,4,5-TCP in an upflow bioreactor with a loading of 2.04 mg/l, day but did not succeed in degrading 2,4,6-TCP and PCP.

Guthrie et al. (1984) observed that a PCP concentration of 5 mg/l was more than 99% removed during anaerobic digestion of sewage sludge in a semicontinuous-flow stirred tank reactor operated at a hydraulic retention time of 10 to 40 days. However, the exent of dechlorination was not reported. Hakulinen and Salkinoja-Salonen (1982) demonstrated the ability of an anaerobic fluidized bed reactor coupled to an aerobic trickling filter reactor to mineralize PCP. In that system, the degradation pathway and the role of the anaerobic reactor were not determined. Experiments in an UASB reactor (Woods et al., 1989) with various chloroaromatic compounds showed that these compounds were partially dechlorinated but never totally mineralized. MCPs appeared to persist as final degradation products. Hendriksen et al. (1992) reported the degradation of PCP in UASB reactors with dichlorophenols as the major intermediates or products. Dechlorination of PCP proceeded via two different pathways. The major route, representing 95% of the added PCP, was initiated by a *para* dechlorination followed by two *ortho* dechlorinations. Nicholson et al. (1992) have shown that batch reactors inoculated with PCP-acclimated sludge were able to remove chlorine from *ortho*, *meta* and *para* positions of PCP to form a mixture of TCPs and DCPs.

In batch experiments using a mixture of the 2-CP-, 3-CP-, and 4-CP-acclimated sludges as the inoculum, PCP was dechlorinated and completely mineralized (Mikesell & Boyd, 1986). Magar et al. (1995) also reported that complete dechlorination of PCP was achieved in a continuously fed fluidized bed reactor and in serum bottles after combining PCP-dechlorinating and 3-CP mineralizing enrichments. Wu and colleagues (1993) have developed methanogenic PCP-degrading granules on a synthetic wastewater containing PCP, acetate, propionate, butyrate, and methanol in an UASB reactor. The reactor was able to treat synthetic wastewater containing 40 to 60 mg of PCP/l and more than 99% of PCP removal was achieved, whereas 3-CP was the only intermediate product

of PCP transformation in UASB reactors treating synthetic wastewater containing low levels of phenol and PCP (Duff et al., 1995).

Purpose of the study

Since its introduction about 15 years ago, the upflow anaerobic sludge blanket (UASB) reactor has been most commonly used for treatment of various industrial wastewater. Recently, many studies have demonstrated that UASB technology is applicable to treating wastewater containing chlorinated aromatic compounds. Although the results of those studies showed that chlorophenols could be degraded in UASB reactors, sludge granules were limited in their abilities to degrade the MCPs resulting from dehalogenation of more-chlorinated phenols (Hendriksen et al., 1992; Mohn and Kennedy, 1992; Wood et al.,1989). Therefore, the purpose of this study was to develop the sludge granules that can dechlorinate evtl. mineralize monochlorophenols. Additionally, it should be investigated whether this cultures were capable of dechlorination of highly-chlorinated phenols or not. The specific objectives of this research were

(1) to acclimate the granular sludges which was previously grown on glucose-containing wastewater to the isomers of monochlorophenols (2-CP, 3-CP and 4-CP).

(2) to examine the physiological and biological factors that affect the dechlorination of MCPs by those acclimated cultures.

(3) to establish and optimize the dechlorination rate of MCPs in UASB reactors.

(4) to determine the kinetic parameters of the MCPs dechlorination.

(5) to examine the potential of those acclimated cultures for transformation of highly-chlorinated phenols.

2 MATERIALS & METHODS

2.1 Laboratory-scale UASB reactor

The primary components of the system are the main reactor compartment, the recycle system, the constant temperature jacket, the feed reservoir, and the liquid and gas effluent stream.

Reactor R 1, used as sludge supply reactor and control system (unacclimated sludge), is a water-jacketed glass column (5.5 cm i.d., 73 cm length). The reactor volume was 2.1 L, and the settler volume was 1.3 L. The

total reactor volume was 3.4 L. The chlorophenol-degrading bioreactor (R 2) was a vertical tank (9 cm i.d.,50 cm length) made of glass and having an operational liquid volume of 2.6 L. Effluent for recirculation was withdrawn slightly below the overflow port and recirculated into the bottom part of the reactor (recycle flow ca. 20 l/h). Fresh feed was pumped into the recirculation stream. Biogas was leaving the reactor through a port at the top and was measured with a wet gas meter. Thermostated water from a water bath was pumped through the temperature jacket surrounding the reactor to maintain the temperature of both reactors at 35°C.

2.2 Sources of granules and inoculum

Granular sludges obtained from a full-scale UASB reactor treating brewery wastewater were used as inocula for reactor R 1 and further fed with glucose containing synthetic wastewater. Glucose-degrading granules from reactor R 1, which were acclimated to tolerate/degrade 2-chlorophenol (2-CP), were used as inocula for reactor R 2.

2.3 Reactor operation

Reactor R 1 was started with glucose containing synthetic wastewater which was prepared from glucose (final concentration 200 mg/l) and from stock solutions of minerals mentioned in Table 2.1. The C:N ratio and N:P ratio for all experiments were kept at 18:1 and 5:1, respectively. The starting biomass concentration was 15 g TS/l. In the start-up period and later on, this reactor was continuously fed with glucose containing synthetic wastewater and was operated under various volumetric organic loading rates (OLR) by varying either the glucose concentration or the hydraulic retention time (HRT). The reactor performance was monitored with daily measurements of the pH in the effluent, of feed rates and of the total gas production. The influent and effluent COD/DOC, gas composition, influent and effluent nitrogen were measured 2-3 times per week.

Reactor R 2 was inoculated with glucose-degrading granules from R 1 which were acclimated to tolerate/degrade 2-CP. The reactor was maintained at a HRT of 5 days and had an initial biomass concentration of 11 g TS/l. The synthetic wastewater for this reactor contained 2-CP (initial conc. 128 mg/l) and mineral medium supplemented with glucose at a fixed concentration of 0.9 g/l. The C:N and N:P ratio were the same as for reactor R 1. A 2-CP concentration of 1 mM was used to start the system and then was increased stepwise to 9 mM. Monitoring parameters (pH, COD, gas composition) were measured as described for reactor R 1, except

influent and effluent nitrogen. Additional parameters such as effluent concentrations of phenol, influent and effluent chloride- and 2-CP concentration were also measured 2-3 times per week.

The pH in both reactors was controlled daily and an appropriate amount of 1 M $NaHCO_3$ was added into the recycle line to maintain the pH at 7.0 - 7.4. The total reactor biomass concentration was estimated from the value of total solid (TS, g/l) of granules which were taken from both reactors for batch experiments.

Table 2.1. Composition of mineral medium (stock solution)

Constituent	Concentration (g/l)	Constituent	Concentration (g/l)
nutrient		***micronutrient*** (Thiel, 1990)	
NH_4Cl	60	$NiSO_4.6H_2O$	5.0
K_2HPO_4	20	$FeSO_4.7H_2O$	5.0
KH_2PO_4	10	H_2BO_3	1.0
		$CaCl_2.2H_2O$	0.5
		$MnCl_2.4H_2O$	5.0
		$ZnSO_4.7H_2O$	1.0
		$Na_2MoO_4.2H_2O$	0.5
		$CuSO_4.5H_2O$	0.05

Note : The amount of micronutrient for each preparation of feed substrate was

0.1 ml/l of substrate

2.4 Analytical procedures

The Chemical Oxygen Demand **(COD)** was determined using the method of Wolf and Nordmann (1977). Organic substances in wastewater samples were oxidized by $K_2Cr_2O_7$ in an acidic solution (H_2SO_4 & H_3PO_4 containing Ag_2SO_4 as catalyst) for 2 hours at 150°C. After oxidation, the green Cr^{3+} ions were measured spectrophotometrically at 615 nm.

The Dissolved Organic Carbon **(DOC)** was determined with a DOC-Analyzer (TOCOR 2, Maihak) using Method 5310 B. Combustion-Infrared Method of *Standard Methods* (APHA,1989).

Ammonia was determined by using Method 4500-NH_3 B. Preliminary Distillation Step of *Standard Methods* (APHA,1989). Ammonia in samples was distilled into a solution of boric acid and was determined titrimetrically with standard H_2SO_4 and a mixed indicator.

Organically bound nitogen **(N_{org})** was determined by using Method 4500-N_{org} B Macro-Kjedahl Method of *Standard Methods* (APHA,1989) after the distillation of NH_4-N.

***Total Solids* (TS)** were determined by evaporating well-mixed samples in a weighed vessel and drying to constant weight in an oven at 103 to 105°C. The organic fraction of TS (oTS or volatile solids) was obtained by subtraction of the mineral content (oxidation at 550 ± 50°C) from the total solids content. (Method 2540 Solids - *Standard Methods*, APHA,1989)

The biogas composition was analyzed with a PACKARD model 427 gas chromatograph equipped with a TCD-detector and 1/8 inch teflon column (1.5 mm x 1.8 m) packed with Poropack N (80-100 mesh). The temperature settings used were as follows: column at 30°C, injector and detector at 100°C. Nitrogen served as the carrier gas at a flow rate of 10 ml/min. Gas samples were withdrawn from the headspace of the tested bottles (in case of reactor, from gas sampling port) using a Pressure Lok® syringe (Precision sampling Corp., Baton Rouge, Louisiana) and injected into the gas chromatograph. Standard methane was injected under the same conditions to determine the concentration in the samples.

Chlorophenols: All chlorophenols were analyzed by high pressure liquid chromatography (HPLC). Frozen samples were thawed and then centrifuged at 14,000 rpm for 10 min. The chlorophenols were measured with a Kontron HPLC under the condition shown in Table 2.2. The sample injection valve (Rheodyne 7161) was fitted with a 20-µl loop.

Chloride concentration: Chloride ion concentration in influent and effluent of 2-CP-degrading reactor was determined by titration using Method 4500-Cl^- C. Mercuric nitrate method of *Standard Methods* (APHA,1989).

Chloride ions in supernatant from tested bottles were determined by ion chromatography (IC). The condition for IC were as follows: column, Alltech Anion/R 4.1 mm i.d.x250 mm; temperature 35°C; eluent, 4 mM p-hydroxybenzoate in 2.5 % (v/v) MeOH, pH 8.5 (adjusted with LiOH); flow rate 2 ml/min; detector GAT-LFD 320 Conductivity Detector.

Table 2.2 HPLC condition for chlorophenols analysis

Pump:	LC pump T-414
Detector:	UV-absorbance (UVIKON 820)
Wavelength:	300 nm (PCP) and 280 nm for others chlorophenols
Integrator:	Shimadzu C-R3A
Column:	Lichrospher 100 RP-18 (5 µm), 4 mm i.d. x 125 mm (Hewlett Packard)
Eluent:	acetonitrile and 5% aqueous acetic acid (7:3,v/v for PCP and 1:1 for others CPs).
Flowrate:	0.6 ml/min

2.5 Chemicals

Most of the chemicals were commercially available products of analytical grade (p.A) purchased from Merck (Darmstadt), Fluka (Neu-Ulm), Sigma (München). Water, methanol, and acetonitrile used in the high performance liquid chromatography (HPLC) analyses were of HPLC grade.

2.6 Batch experiments

For each batch experiment, unacclimated sludge (from R 1) or acclimated sludge (from R 2) (about 8-10 g TS/l) was mixed with sterile phosphate buffer pH 7.5 and the respective phenolic substance in a 120 ml serum bottle to give a total volume of 50 ml, except where stated otherwise. All experiments were done in duplicate. Serum bottles were prepared in an anaerobic chamber. After displacing the head space air with N_2 gas, the serum bottles were tightly capped with a rubber stopper and incubated at 35°C on a rotary shaker.

2.6.1 Preliminary test

To determine the ability of the sludge to degrade monochlorophenol, unacclimated granular and suspended sludge were incubated with 1 mM of 2-CP in 20 ml phosphate buffer, pH 7.5, at 35°C. Methane production was monitored for over 40 days and compared with control samples that did not contain 2-CP.

2.6.2 Determination of the anaerobic biodegradation potential and toxicity of each monochlorophenol (2-CP, 3-CP & 4-CP) on unacclimated sludge

1. Biochemical Methane Potential (BMP) test

This method was slightly modified from that employed by Owen *et al*. (1979). Granular sludge from an UASB reactor fed with glucose containing synthetic wastewater was used as an inoculum . Various concentrations of MCP were added to the 120 ml serum bottles. A control without MCP was incubated for comparison. Total gas production and the MCP concentration were measured over 30 days.

2. Anaerobic Toxicity Assay (ATA)

A methane production test, similar to the method of Owen et al. (1979), was used to estimate the overall inhibition of methane production. The 120 ml serum bottles were filled with 48 ml of the mixture of mineral medium, unacclimated sludge and various concentrations of MCP. A spike of 2 ml of acetate-propionate solution was introduced into the serum bottles, resulting in an acetate and propionate concentration of about 25 mM and 10mM, respectively. A control sample without toxicant was also included. Methane production was subsequently monitored every 24 h for 5 days.

2.6.3 Determination of the kinetics of 2-CP dechlorination

Acclimated granular sludge (from R 2) was incubated with phosphate buffer pH 7.5 at 35°C. The 2-CP was added from a stock solution (100 mM) to give a final concentration ranging from 0.5 to 5.0 mM. Glucose (5 mM or 0.9 g/l final conc.) was added to all bottles, except in the experiment for testing the effect of glucose addition on 2-CP dechlorination. Liquid samples were periodically taken and monitored for 2-CP depletion. The dechlorination rates were calculated from the slope of the 2-CP depletion curve (between 1-6 h). The half-saturation constant (K_m) and the maximal dechlorination rates (V_{max}) were determined according to Lineweaver-Burk (1934).

2.6.4 Determination of the kinetic parameters for glucose degradation

Acclimated granular sludge (from R2) and unacclimated granules (from R 1) were incubated with various concentration of 2-CP (range from 0-5 mM) and a definite concentration of glucose (i.e. 500, 1000, or 2000 mg/l). The glucose degradation rates were calculated from the slope of the glucose depletion curves. The inhibition constants (K_i and K_{ii}), K_m and V_{max} were estimated by the graphical method, in accordance to Dixon (1953) and Cornish-Bowden (1974).

2.6.5 Determination of glucose concentration

The PABAH-reagent (Wozniewski, 1990) consisted of 2 solutions as shown in Table 2.3. One part of solution A was mixed with 9 parts of solution B before being used. The mixture of 0.2 ml sample and 4.8 ml test

solution was boiled for 6 min. in a water bath and was measured spectrophotometrically at 410 nm. A standard curve for 0-150 mg glucose/l was prepared.

Dinitrosalicylic acid-reagent (Miller, 1959) was used to avoid the error in dilution of sample when high concentration of glucose (>1000 mg/l) were determined. The composition of Dinitrosalicylic acid-reagent is shown in Table 2.4. The mixture of 0.25 ml sample, 0.25 ml H_2O bidest. and 1.5 ml reagent was boiled in a water bath for 5 min. After cooling, 3 ml H_2O bidest was added and color intensity of the solution was measured spectrophotometrically at 550 nm. A standard curve was prepared for 0-700 mg glucose/l.

Table 2.3 Composition of PABAH-reagent

Solution A	4-Hydroxy-benzoic acid hydrazide HCl (0.5 M)	7.61 g ad. 100 ml
Solution B	NaOH $CaCl_2$ Na-citrate	20 g 1.1 g 14.7 g ad. 1000 ml H_2O

Table 2.4 Composition of Dinitrosalicylic acid-reagent

Dinitrosalicylic acid	10 g
Phenol	2 g
NaOH	10 g
Na/Ka-tartrate	200 g
Na_2SO_3	0.5 g
H_2O bidest	ad 1000 ml

2.6.6 Determination of biosorption of PCP to anaerobic granular sludge

Preliminary sorption experiments were conducted by contacting 2-CP-degrading granules in serum bottles with 40 mg/l PCP. The equilibrium isotherm was evaluated from the results of the sorption experiment, in which anaerobic granules were contacted with concentrations of PCP ranging from 0.4 to 16 mg/l. Control samples containing dilution medium but no biomass were also run to evaluate the sorption to the glass surface (not due to biomass). All serum bottles were agitated on an orbital shaker for 24 hours at 35°C. One ml from all serum bottles was removed every hour until 8 th h and 24 th h for analysis of the PCP concentration in controls and tested samples by HPLC, using a reversed-phase column (Lichrospher 100, RP-18) and a dioade array

detector. Wavelengths for detection were 280 and 300 nm. Acetonitrile and 5 % of acetic acid in the ratio of 70:30 were used as a mobile phase with a flow rate of 1.0 ml/min.

3 RESULTS

3.1 Performance of an UASB reactor for the treatment of glucose containing synthetic wastewater (reactor R 1)

3.1.1 COD and TKN removal efficiency

A UASB reactor (R 1), inoculated with granular sludge from a full-scale UASB reactor treating brewery wastewater, was fed with an artificial glucose-containing wastewater to give a HRT of 8 h. The HRT was changed to 24 h after the pellets were partially inactivated by high temperature (failure of the thermostat). When the reactor removed over 80% of the total COD from the wastewater, the organic loading rate was increased stepwise. Figure 3.1 depicts the relationship between removal efficiency (COD and TKN) and organic loading rate.

At a constant HRT of 8 h, the glucose concentration in wastewater was varied from 1 to 5 g/l, corresponding to a organic loading rate of 5-15 g COD/l, d. A COD removal efficiency ≥ 90 % was achieved when the reactor was operated at organic loading rates lower than 9 g COD/l, d. The COD removal efficiency decreased to 77 % when the organic loading rate was increased to 12 g COD/l, d.

During the experiment, the temperature of the reactor was accidentially heated 35 °C to 80°C for about 4-5 hours, due to the breakdown of the thermostat. Flotation of granules (due to increased CO_2 evolution by a temperature shift of the equilibrium $CO_3^{=}$ $HCO_3^{=}$ CO_2) was observed immediately after the occurence of shock heating. In an attempt to restore the biological activity in the system and to reduce the loss of granules, the upflow velocity in the reactor was reduced from 8.4 to approx. 5 m/h and medium addition was omitted until the quantity of floating granules was reduced significantly. The recovery of granules began on the fifth day after shock heating. The reactor was then fed again and the organic loading rate was slowly increased.

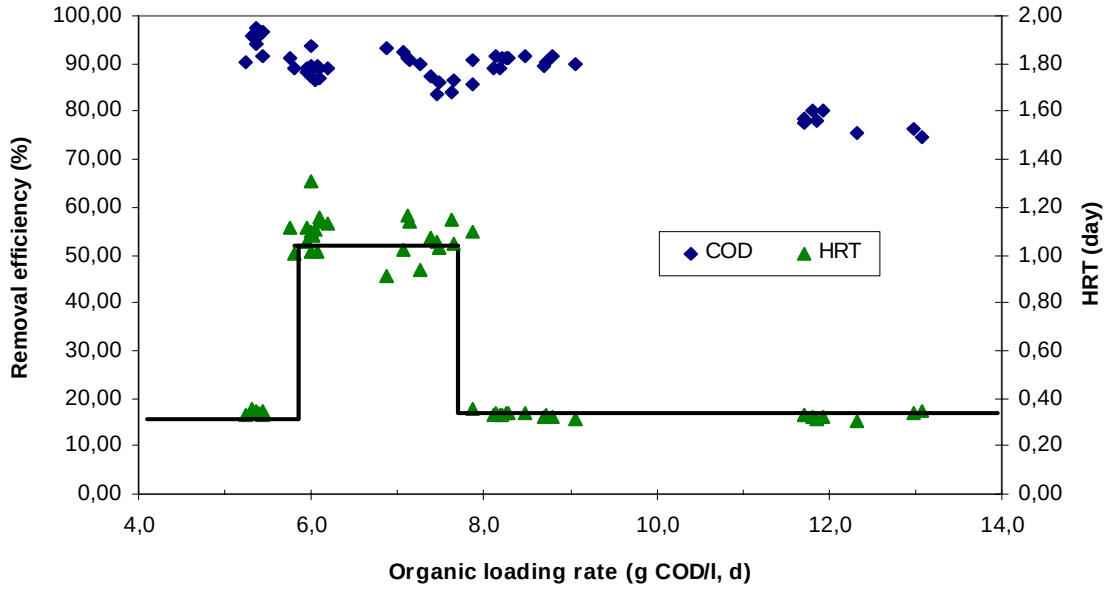


Fig. 3.1 HRT and total COD removal efficiency of reactor R 1 at different organic loading rates.

After the heat shock the effectiveness of granules for anaerobic digestion was greatly diminished. A COD removal efficiency of 90 % was hardly attained even at a loading rate as low as 5 g COD/l, d. The granules began to clump and stick together when the glucose concentration in the medium was higher than 2 g/l. Consequently, the upflow velocity of the reactor was raised to its preceding value of 8.4 m/h to enhance the mixing in reactor and the HRT was increased to 15 h in order to reduce the loading and to improve the removal efficiency. However, a satisfying removal efficiency (over 80 %) was not achieved until the HRT of the reactor was increased to 24 h. The system could remove approx. 90 % of the COD in wastewater at the provided loading rate between 6-8 g COD/l, d. When the organic loading was higher than 9 g COD/l, d the removal efficiency dropped drastically to around 50 % and only half of the expected methane was produced (data not shown). Many fatty acids e.g. acetate, propionate, butyrate were found in the effluent indicating that the reduction of removal efficiency was the result of an imbalance between acid formers and acetogenic and methane bacteria in the system.

No nitrogen was removed during anaerobic degradation, except for that portion used for growth of the bacteria.

3.1.2 Methane production

The change of the methane production rate during the experiment is shown in Figure 3.2. Anaerobic digestion of sugars like glucose can theoretically yield about 0.35 l_{STP} of methane per 1 g of COD, as derived from the Buswell equation (Eq. 1) or the conversion stoichiometry.

$$C_nH_aO_b + (n - \frac{a}{4} - \frac{b}{2})\,H_2O \Rightarrow (\frac{n}{2} - \frac{a}{8} + \frac{b}{4})\,CO_2 + (\frac{n}{2} + \frac{a}{8} - \frac{b}{4})\,CH_4 \quad \text{..... (1)}$$

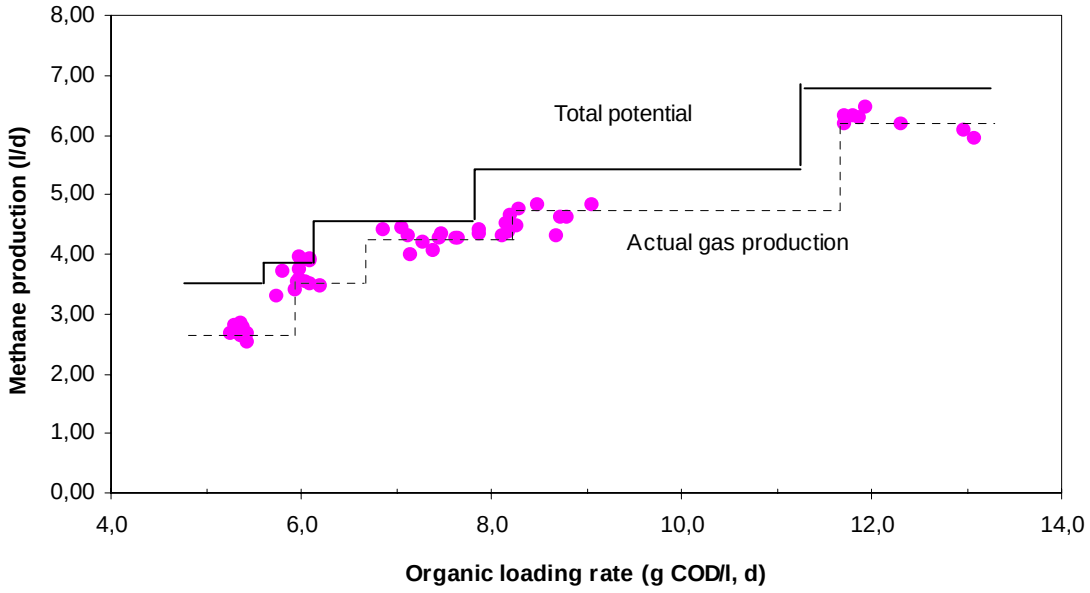


Fig. 3.2 Actual and potential methane production from reactor R 1 at different organic loading rates.

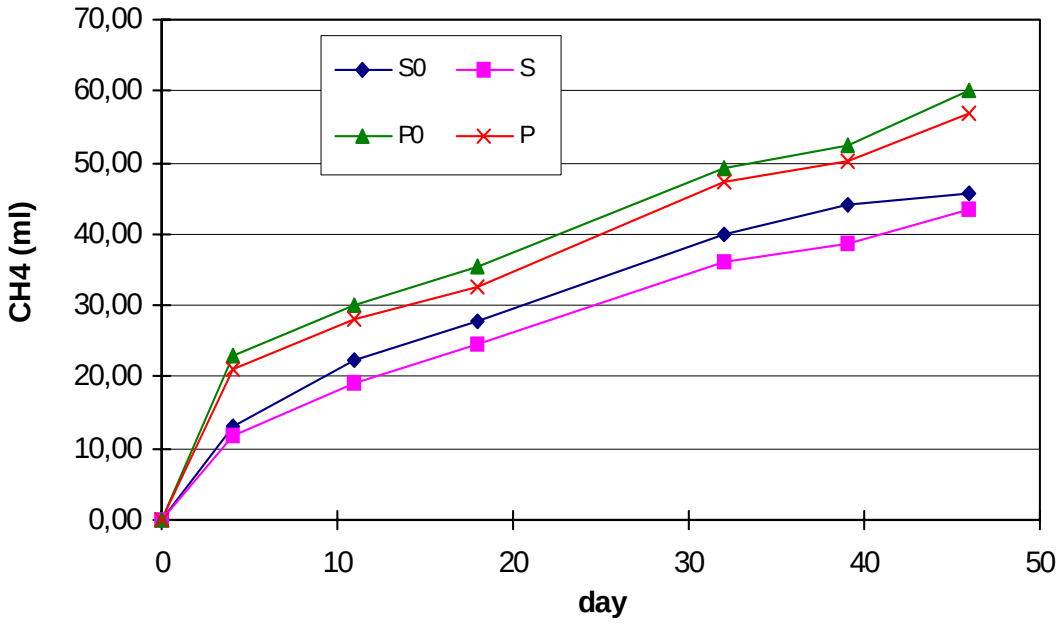
The total potential of the methane production rates, calculated theoretically from the COD load was drawn as a solid line in Figure 3.2. The actual methane production at each organic loading rate was 10-27 % less than the theoretical value. The difference between actual methane production and theoretical value was only 7-10 % when the HRT of the reactor was increased to 24 h. Usually, gas from anaerobic digestion of mixed substrates is composed of 70-85 % methane, 15-30 % carbon dioxide and a small amount of hydrogen sulfide, H_2 and other gases. Methane measurement of the effluent gases from reactor R 1 revealed that the biogas contained about 65 % methane, as typically obtained, when carbohydrates were fermented.

3.2 Acclimation and enrichment of chlorophenol-degrading granules

3.2.1 Preliminary test

The ability of unacclimated sludge to degrade monochlorophenol was examined in 120 ml serum bottles by incubating glucose-degrading sludge pellets from reactor R 1 with 1 mM of 2-chlorophenol (2-CP) as initial concentration in 20 ml phosphate buffer, pH 7.5, at 35°C. The control samples that did not contain 2-CP were incubated under the same condition. The 2-CP was selected as a representative of monochlorophenols because it was reported to be degraded by undefined cultures in several systems (e.g., Boyd & Shelton, 1984; Gibson & Suflita, 1986; Dietrich & Winter, 1990; Mohn & Kennedy, 1992; Flora et al., 1994) and even by a pure culture, strain 2CP-1 (Cole et al., 1994).

Two forms of unacclimated inoculum, granular sludge from R 1 and suspended sludge from a sewage digester, were used in this test and methane production was monitored for over 40 days. Within 46 days the



methane amount in granular sludge (P) and suspended sludge (S)

Fig. 3.3 Methane production of suspended (S) and granular sludge (P), incubated at 35°C with 1 mM of 2-CP in 20 ml phosphate buffer, pH 7.5. The methane production of the control samples that did not contain 2-CP (S_0, P_0) was also measured.

samples has reached 54 and 43 ml, respectively (Fig. 3.3). The result showed that both sludge sources retained their potential to form methane in the presence of toxic substances like 1 mM 2-CP. Apparently no methane was formed from 2-CP.

3.2.2 Anaerobic biodegradation potential of unacclimated sludge for different substituted monochlorophenols (2-CP, 3-CP, 4-CP)

From methane measurement of the previous experiment, it could not be shown whether 2-CP was transformed or degraded by these sludges. Therefore, the quantitative determination of chlorophenol or phenol concentrations by high performance liquid chromatography (HPLC) was established in order to verify a biodegradation of chlorophenols or a biotransformation by reductive dehalogenation.

To examine the anaerobic biodegradation potential of unacclimated sludge for different monochlorophenols (2-CP, 3-CP, and 4-CP) or the inhibitory effect of these substances, glucose-degrading granules were incubated with various concentrations of each MCP ranging from 0.5 mM to 10 mM. A control without toxicant was included as a basis for comparison. Total methane production and MCP disappearance were monitored for 9 weeks.

Cumulative methane production from each sample is shown in Figure 3.4 (a-c). For each MCP, gas production gradually decreased as the concentration of the three MCPs increased. Only little methane was produced in all assays (not more than 40 ml/g TS) when the MCP concentration was over 2 mM. While for each MCP a similar methane production profile was obtained, the biotransformation of the MCPs differed significantly. The transformation of 3-CP occurred mostly in the first 2-3 weeks of incubation. After that the concentration of 3-CP in each sample was more or less unchanged whereas 2-CP and 4-CP were further transformed until the 5^{th} week. Table 3.1 shows % transformation of each MCP by the glucose-degrading granules after day 35 of incubation.

Table 3.1 Percentage of each MCP transformed by glucose-degrading granules until day 35 of incubation.

MCP concentration	**transformation (%)**		
	2-CP	***3-CP***	***4-CP***

0.5 mM	100	43.70	25.60
1.0 mM	100	38	21.40
2.0 mM	34.30	30.30	15.60
5.0 mM	22.54	14.86	7.28
7.5 mM	14.95	11.76	7.28
10.0 mM	10.32	5.84	5.50

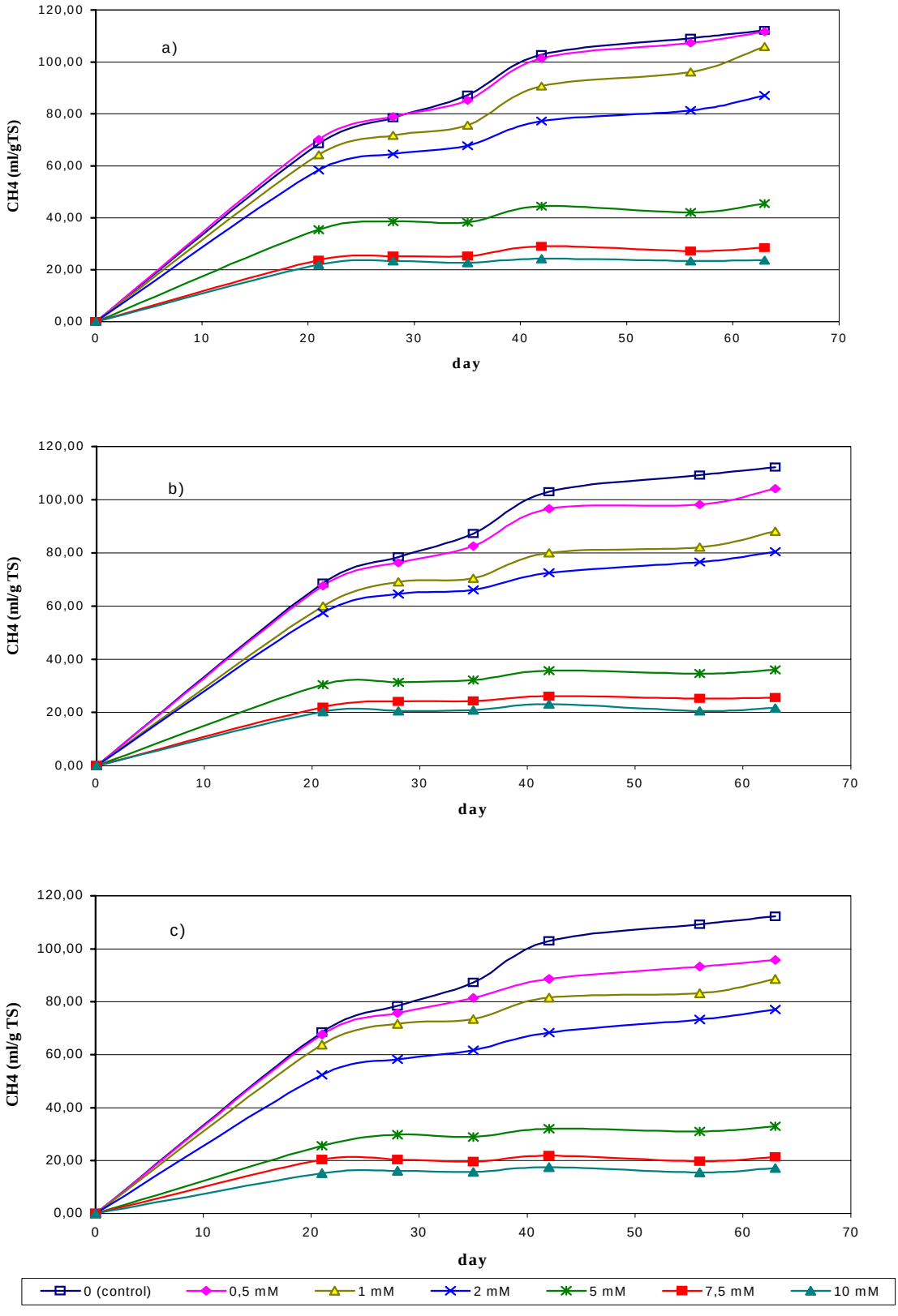


Fig. 3.4 Total methane production of granular sludge from an UASB reactor fed with glucose containing synthetic wastewater, which was supplemented with various concentrations of 2-CP (a), 3-CP (b) and 4-CP (c) in 120 ml serum bottles.

From the results of Table 3.1, it is apparent that the position of the chlorine substituent affects the degradation or dehalogenation of MCP. 2-CP was transformed to a higher degree than 3-CP and 4-CP. Especially at the concentration of 0.5 and 1 mM, 2-CP and phenol, the transformation product from reductive dechlorination, was completely degraded within 35 days whereas 3-CP and 4-CP were transformed only to an extend of 38-44 % and 21-26 %, respectively.

3.2.3 Toxicity of monochlorophenols on unacclimated sludge

Anaerobic toxicity assays (ATA) were performed to evaluate the toxicity of various concentrations of monochlorophenols. The initial concentations of MCP were 0.5, 1, 2, 5, and 10 mM. As can be seen from Figure 3.5 a-c, toxicity increased with increasing dose of toxicant until gas production ceased (100 % inhibition). At the MCP concentrations of ≥ 5 mM, methane production was more than 60 % inhibited. The close agreement between inhibition curves of each concentration of 3-CP (Fig. 3.5 b) and 4-CP (Fig. 3.5 c) at different test times indicated that there was no recovery from the toxic impacts during the incubation period. On the other hand, the inhibition curves of 2-CP at concentrations up to 2 mM (Fig. 3.5 a) showed a slight recovery from the toxic effect of 2-CP which improved with time. For example at a concentration of 2 mM, methane production was about 40 % inhibited in the first 20 hours of the incubation but after 5 days (120 hrs.) the inhibition was only 23 %. The inhibitory effect of the chlorophenols was about 10 % decreased when the old samples were supplemented with acetate-propionate solution and MCP again. From this result, it can be assumed that degradation of MCP had occured or that the organisms did acclimate or adapt to the presence of that chlorophenol isomer. This phenomenon could obviously be seen in the sample which was incubated with 5 mM 2-CP after new acetate-propionate solution and 2-CP were added

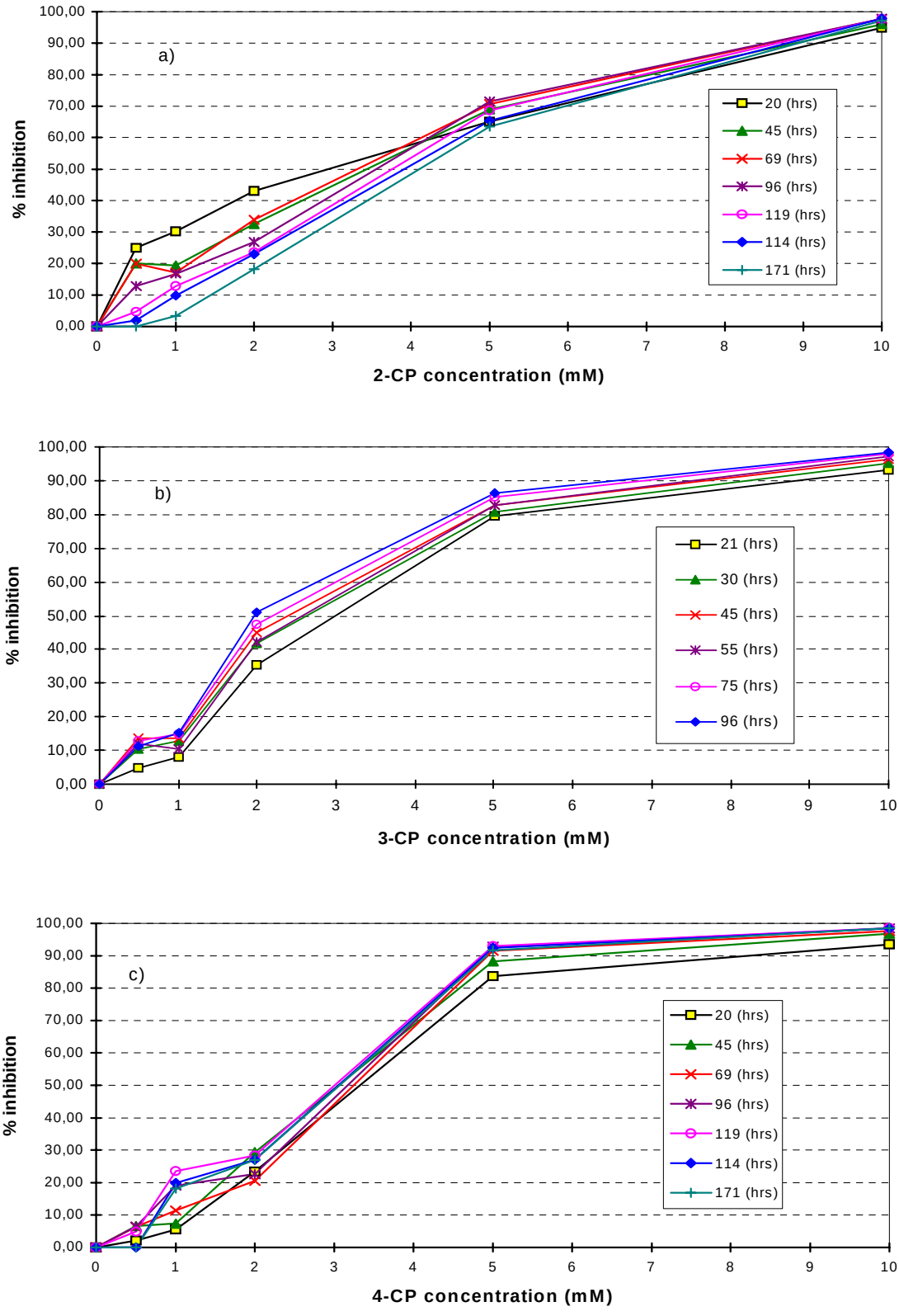


Fig. 3.5 Inhibition of methane production from an acetate-propionate solution with increasing incubation time, when unacclimated granular sludge was incubated with 5 different concentrations of (a) 2-CP, (b) 3-CP and (c) 4-CP at 37°C.

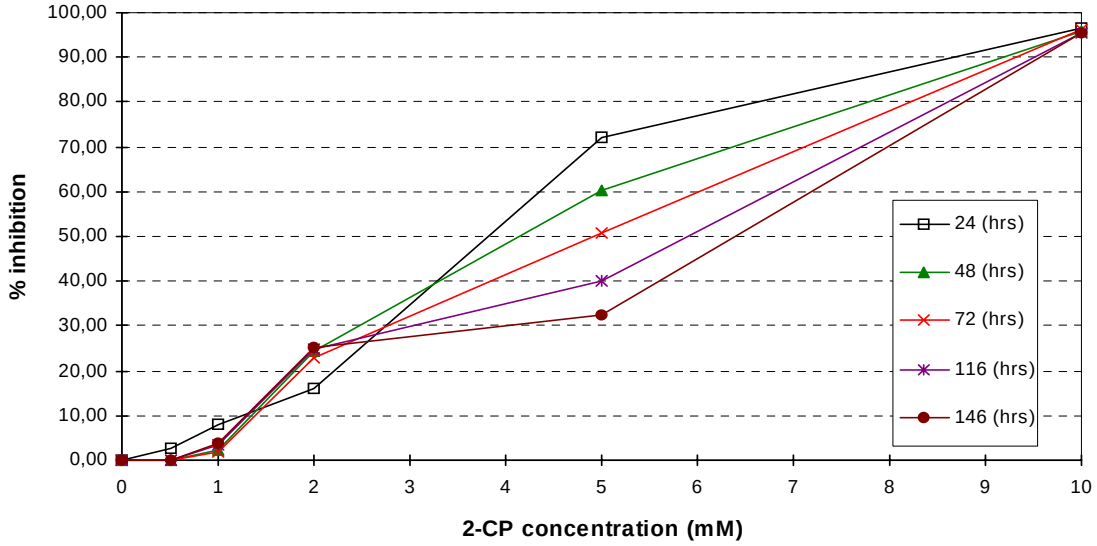


Fig. 3.6 Inhibition of methane production from an acetate-propionate solution by 2-CP in unacclimated granular sludge cultures after substrate and 2-CP were added a second time.

(Fig. 3.6). The inhibition of methane production was 50 % less than in the preceding incubation. In Figure 3.7 the inhibition after 48 h of incubation for different concentrations of each MCP of methane production was compared. It seemed likely that 3-CP had the most toxic effect on methane production among the three isomers of MCP.

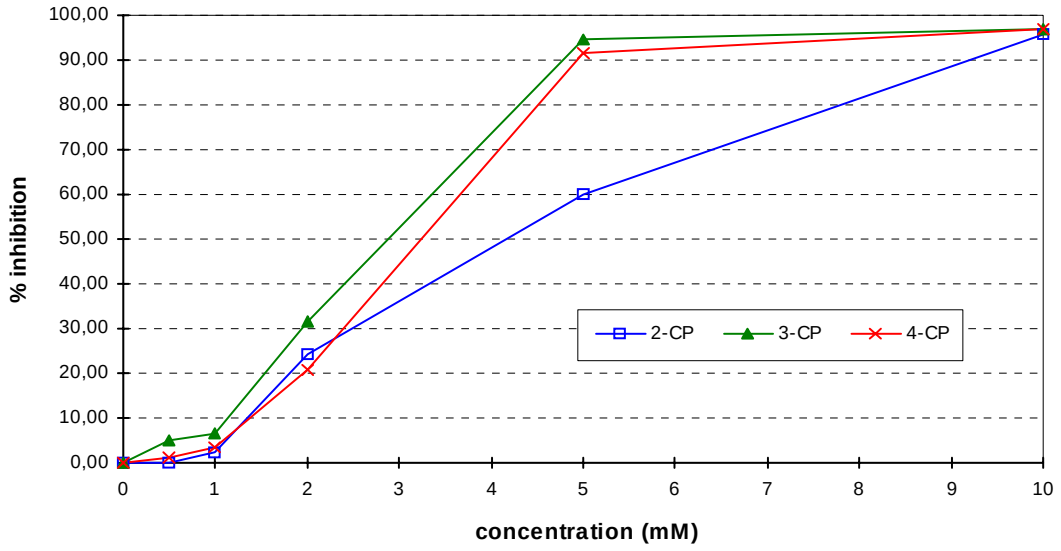


Fig. 3.7 Inhibition of methane production from acetate-propionate solution by each monochlorophenol in unacclimated granular sludge cultures after incubation for 48 hours.

3.2.4 Effect of organic substrates on dechlorination of monochlorophenol

Results from preceding experiments demonstrated that 2-CP, 3-CP, and 4-CP at concentration up to 1 mM could either be degraded by unacclimated sludge or their effect on methane production from other substrates was relatively low (less than 10 % inhibition of gas production). In addition, there was almost no difference in degradation and the inhibitory pattern of methane production for each MCP between 0.5 mM and 1 mM. Thus, 1 mM of MCP, a safe level based on the results of the toxicity assays, was selected to use as an initial concentration for selection of MCP-degrading granules and as a basic concentration for further experiments.

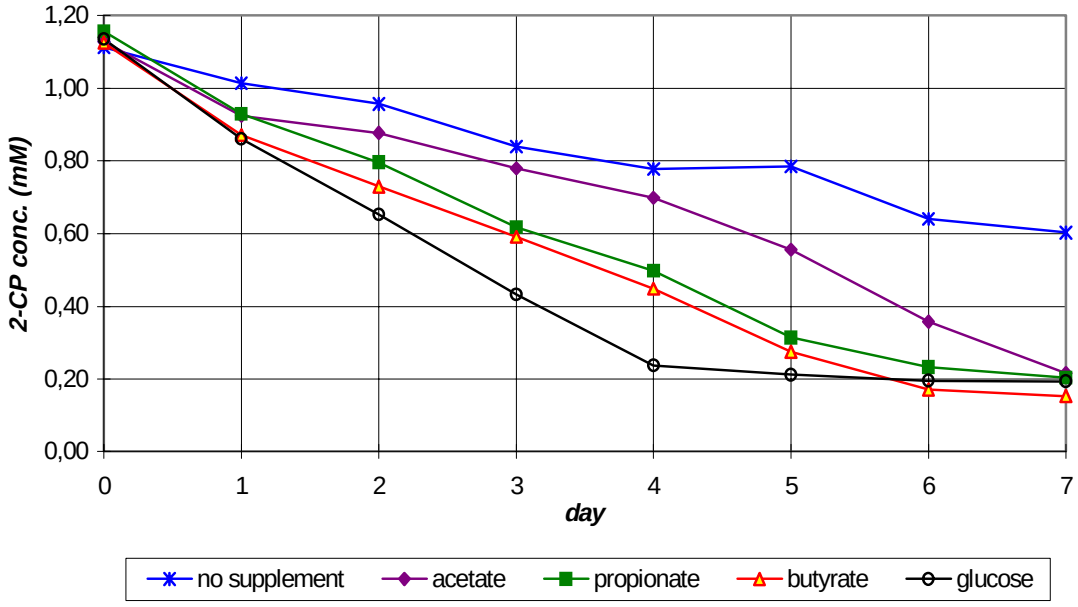


Fig. 3.8 Effect of organic substrates on the dechlorination of 2-chlorophenol.

The stimulatory effect of organic substrates on anaerobic dehalogenation of haloaromatic substances had been reported in many studies (Kuhn et al., 1990; Nies & Vogel, 1990; Gibson & Suflita, 1990; Hendriksen et al., 1992). For this reason, it would be useful to study whether supplemental organic substrates also have positive influence on acclimatization to dechlorination of MCP in granular sludge or not. Glucose-degrading granules were dosed with 1 mM 2-CP, 3-CP or 4-CP and incubated anaerobically together with 2 mM of acetate, propionate, butyrate, or glucose. A control sample (no additional carbon supplement) was similarly incubated. Methane production and depletion of both, 2-CP and the organic substrates, were analysed. After 7 days of incubation, 2-CP in all samples was almost completely dechlorinated (Fig. 3.8). All of the 4 different organic substrates enhanced the dechlorination rate of 2-CP. Glucose had the greatest stimulatory effect. The dechlorination in the samples which were dosed with 3-CP or 4-CP occured only to an extend of less than 10 %, independently of whether any of the organic substrates was amended or not. For further experiments glucose was

used as a carbon source to stimulate the dechlorination of 2-CP, 3-CP and 4-CP during acclimatization of glucose-degrading granules to each MCP. Unfortunately, the transformation of 3-CP and 4-CP in the enrichment cultures did not improve after 3 months of acclimation time. Hence, only an enrichment culture which was acclimated to 2-CP was available for further experimentations.

3.3 Optimal conditions for operating a continuous UASB reactor for treatment of a 2-CP containing synthetic wastewater

3.3.1 Optimum concentration of glucose amendment

Five concentrations of glucose (2 mM, 5 mM, 10 mM, 15 mM, and 20 mM) were supplemented to find out the most suitable concentration for dechlorination of 1 mM of 2-CP. As shown in Figure 3.9, the dechlorination rates of 2-CP for all concentrations of glucose were not significantly different, especially in the first 8 hours of incubation. The control sample which obtained no glucose was incubated in parallel to confirm the stimulatory effect of glucose on dechlorination.

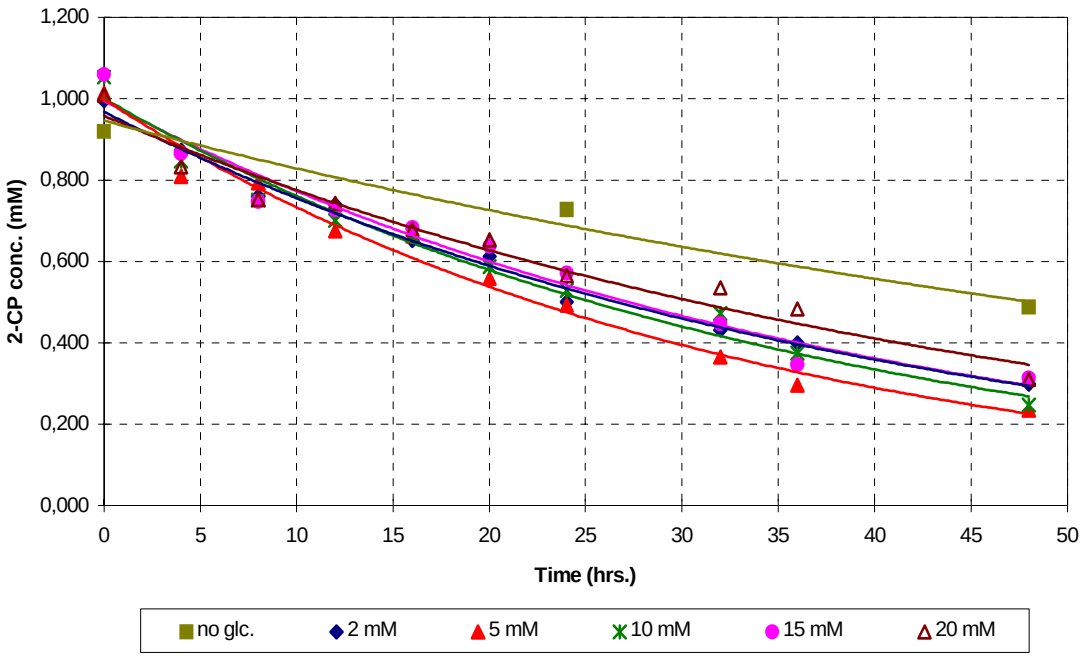


Fig. 3.9 Dechlorination of 2-CP in the presence of various glucose concentrations.

By using the non-linear regression method, the transformation rates of 2-CP could be compared. The initial velocity of 2-CP disappearance increased in the sequence from 5 mM > 10 mM > 15 mM ≥ 2 mM

> 20 mM, and rates calculated for 4 hours of incubation were 0.48, 0.43, 0.41, 0.38, and 0,32 µM/min, respectively. Considering the rates of dechlorination, 5 mM of glucose was the optimal concentration for dechlorination of 1 mM of 2-CP. The good adaptation of this enrichment culture to 2-CP can also be seen from Figure 3.9, since 2-CP at the same concentration of glucose (control and 2 mM) was more than 50 % dechlorinated within 48 hours, opposed to only 15 - 40 % in the experiment shown in Figure 3.8.

3.3.2 Optimal pH for dechlorination

During determination of the optimal glucose concentration for 2-CP degradation, it was found that dechlorination was strongly dependent on the pH of the medium. One mM of 2-CP was incubated with various concentrations of glucose ranging from 5 mM to 20 mM in two different buffer systems, i.e. bicarbonate (initial pH = 8.5) and phosphate buffer (initial pH = 7.8). The effect of pH on the dehalogenation of 2-CP is shown in Figure 3.10 (a-c).

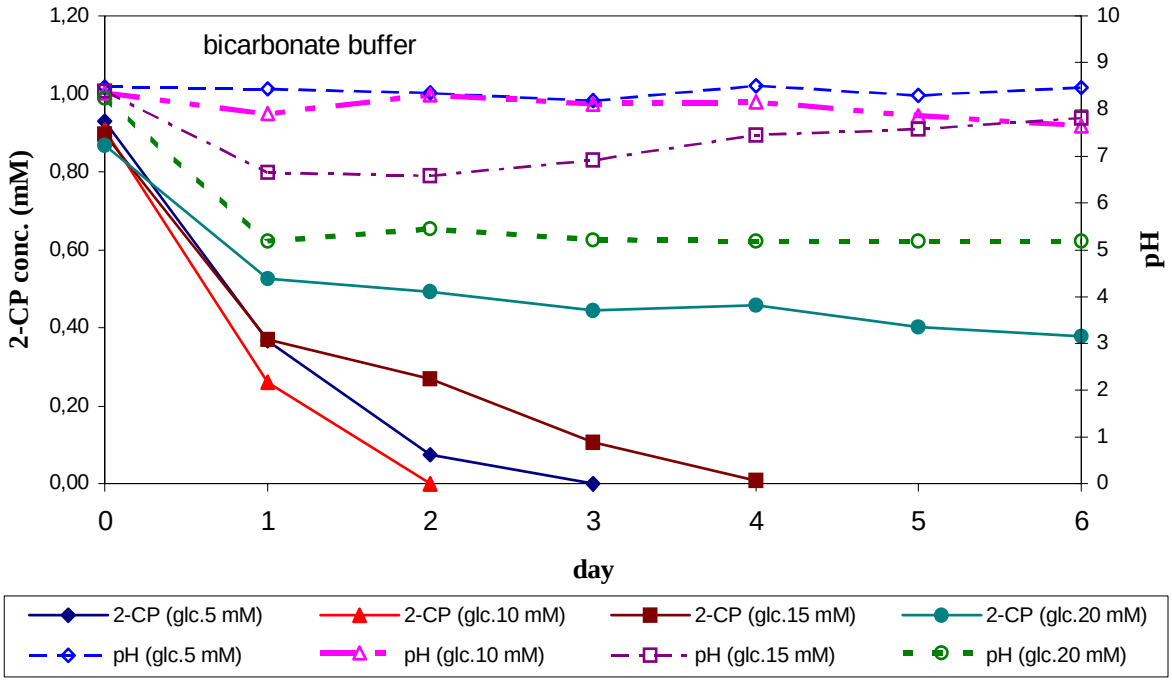


Fig. 3.10 a. Effect of pH on the dechlorination of 1 mM 2-CP in bicarbonate buffer when buffer capacity was not sufficient.

2-CP was completely dechlorinated within 2-4 days when the pH was between 7.0-8.5 (Fig. 3.10 a, b). Dechlorination of 2-CP could also occur when the pH was between 6.5-7.0 but the cultures required more time (5-8 days) for a complete dechlorination of 2-CP (Fig. 3.10 c). It should be noted that 2-CP in samples with a

pH-value below 5.0 could no more be dechlorinated, eventhough the pH of the system was raised to 8.0 (Fig. 3.10 c, arrow). It was assumed that the organisms which have the capability for dechlorination were damaged during 5 days of incubation in low pH condition. The decrease of the pH in the system due to the degradation of glucose indicated that the buffer capacity of the system was not sufficient and then affected the dechlorination of 2-CP. Once the pH of the system was kept stable (i.e. sufficient buffering capacity, Fig. 3.10 b.), there was no marked difference on the dechlorination rates of 2-CP at each concentration of glucose.

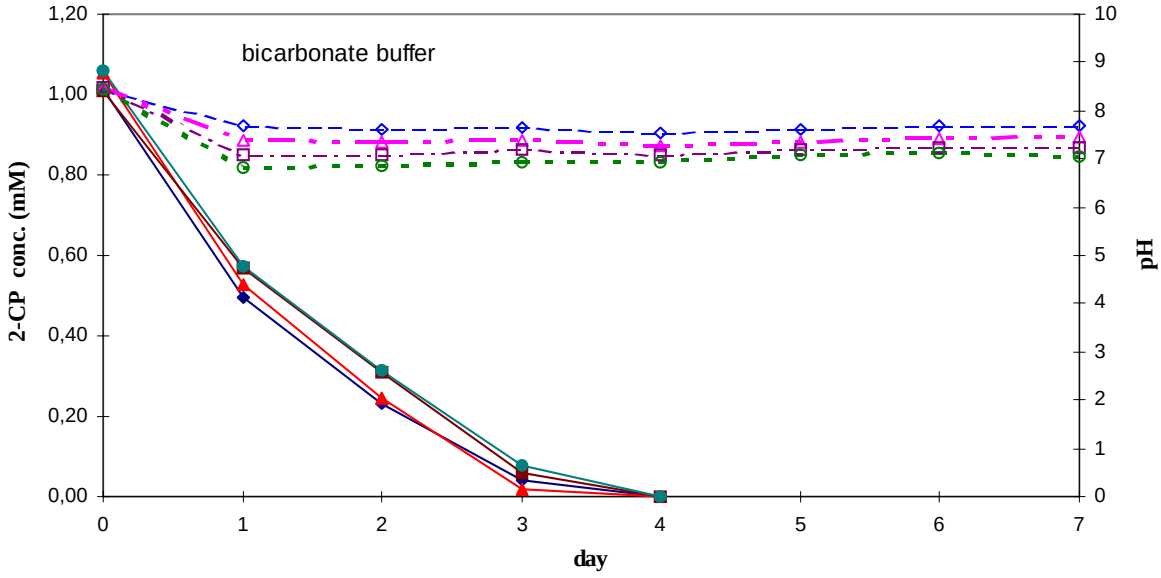


Fig. 3.10 b. Effect of pH on the dechlorination of 1 mM 2-CP in bicarbonate buffer when the buffer capacity was sufficient. All symbols are the same as in Fig. 3.10 a.

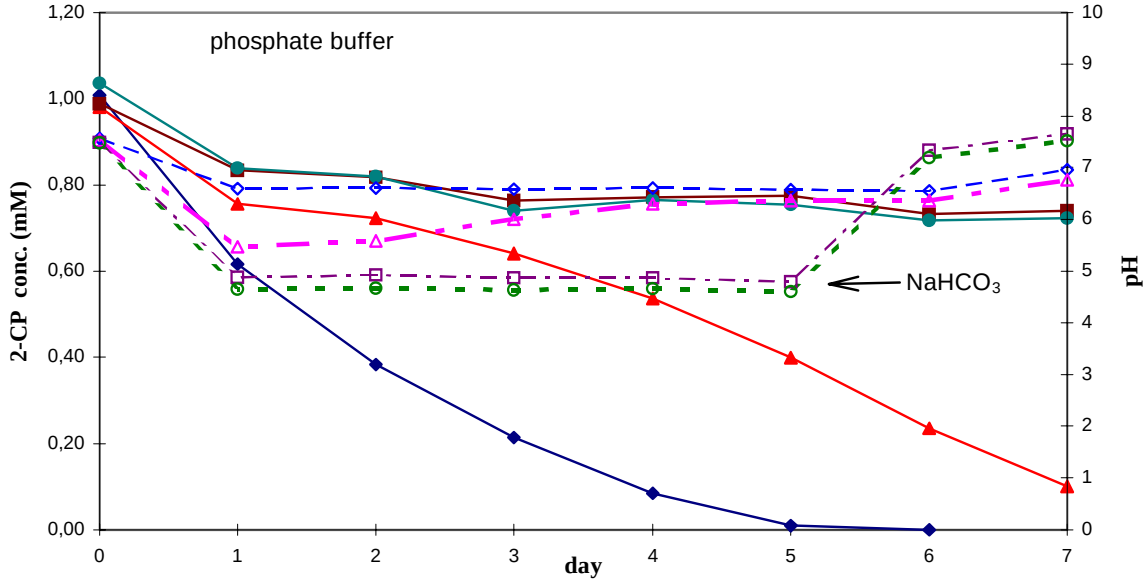


Fig. 3.10 c. Effect of pH on the dechlorination of 1 mM 2-CP in phosphate buffer when the buffer capacity was not sufficient. All symbols are the same as in Fig. 3.10 a

3.4 Performance of the UASB reactor for treatment of 2-CP containing synthetic wastewater (reactor R 2)

The reactor was initially fed 900 mg/l (5 mM) of glucose and 128 mg/l (1 mM) of 2-CP and was operated at a HRT of 5 days to ensure the transformation of 2-CP in the reactor. The flowrate of the recycle stream was maintained at 20 l/h throughout the experiment, resulting in an upflow velocity of approx. 3.1 m/h. Figure 3.11 illustrates the performance of reactor R 2. In Fig. 3.11 a. phenol, 2-CP concentration and HRT, in Fig. 3.11 b. 2-CP loading and % removal, in Fig. 3.11 c. the corresponding COD loading rate and % removal and in Fig. 3.11 d. the total gas production and % methane in biogas are shown.

3*.4.1 Response to hydraulic loading shock*

About 20 days after start up, the 2-CP removal efficiency had reached over 97 % and phenol, an intermediate from dechlorination, was not detectable in the effluent even though the 2-CP concentration in the influent was increased to 1.5 mM (Fig. 3.11 a, b). On day 90 with a stable 2-CP loading rate of 50 mg/l, d the HRT of the system was lowered from 4.5 day to 2.8 day to see whether the high removal efficiency of the startup period could be maintained. The 2-CP removal efficiency dropped to 75 % and seemed difficult to recover to the pre-shock level while the COD removal efficiency was only decreased by 12% (from 90 to 78 %.). Thus, on day 120 the HRT was raised to 4.6 days again, at which 2-CP removal efficiency recovered gradually. Nevertheless, the full recovery of chlorophenol removal activity was not achieved even after 50 days at low 2-CP loading rates as in the startup period.

3.4.2 Response to temperature shock

On day 179, the reactor experienced a temperature shock for about 4-5 hours (from 35°C to 80° C, due to a failure of the thermostat). This temperature change adversely affected

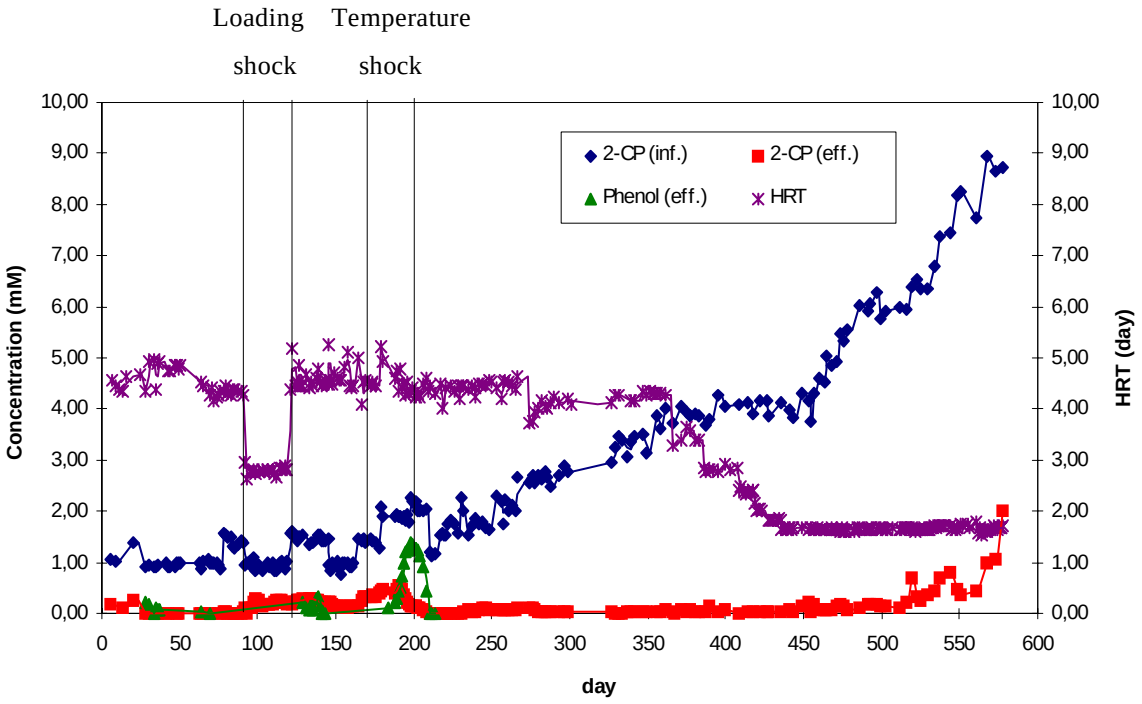


Fig. 3.11 a. Performance of reactor R 2 : Phenol, 2-CP concentration and HRT.

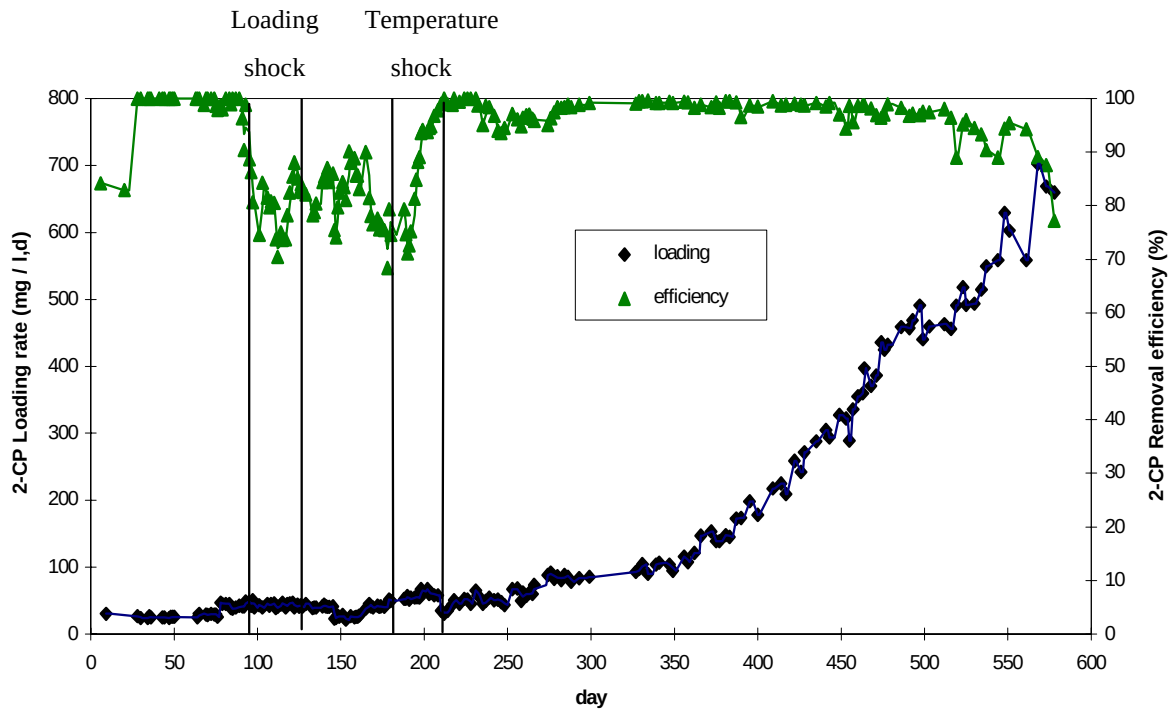


Fig. 3.11 b. Performance of reactor R 2 : 2-CP loading rate and % removal.

bioactivity the 2-CP degrading granules especially the COD removal rate. As illustrated in Figure 3.11 (b, c, d), the COD removal efficiency dropped readily from 85 to 22 % whereas the chlorophenol removal efficiency was only reduced by 20 %. The biogas production rate was reduced by more than 50% and the proportion of methane in biogas dropped from 60 to 32 %. The concentration of 2-CP in the effluent was increased and coupled with the appearance of phenol which was later degraded when the system was recovering. It took about 40 days for the 2-CP concentration in effluent, COD removal efficiency and biogas production rate to recover to the levels before the temperature shock.

3.4.3 COD removal, 2-CP removal and biogas production

After a full recovery of bioactivity of the pellets was accomplished, the 2-CP loading rate was gradually increased by either an increasing concentration of 2-CP in the influent or the reduction of the HRT (Fig. 3.11 a). A removal efficiency of 2-CP over 95 % was achieved for nearly 10 months during days 220-510 while the 2-CP loading rate was steadily raised from 50 to 500 mg/l, d (Fig. 3.11 b). A further increase of the 2-CP loading rate reduced the chlorophenol removal efficiency of the system and the 2-CP concentration in the effluent slowly increased. However, no trace of phenol was found in the effluent during this time. At the termination of the experiment the 2-CP loading rate was 700 mg/l, d, and the removal efficiency dropped to 76 %. 2-CP in the effluent had reached the concentration of 2 mM.

Since the concentration of glucose in the influent was kept constant at 0.9 g/l throughout the experiment, at a constant HRT the increasing of the organic loading rate was achieved by increasing the 2-CP concentration in the influent. As illustrated in Figure 3.11 c., the COD removal efficiency of the system after the temperature shock proceeded in the same manner as the 2-CP removal efficiency. An average COD removal efficiency of 85 % was attained during days 220-510 while the organic loading rate was increased from 0.38 to 1.60 g COD/l, d. The COD removal efficiency was steadily decreasing from 85 % to 70 % when the organic loading rate was further increased.

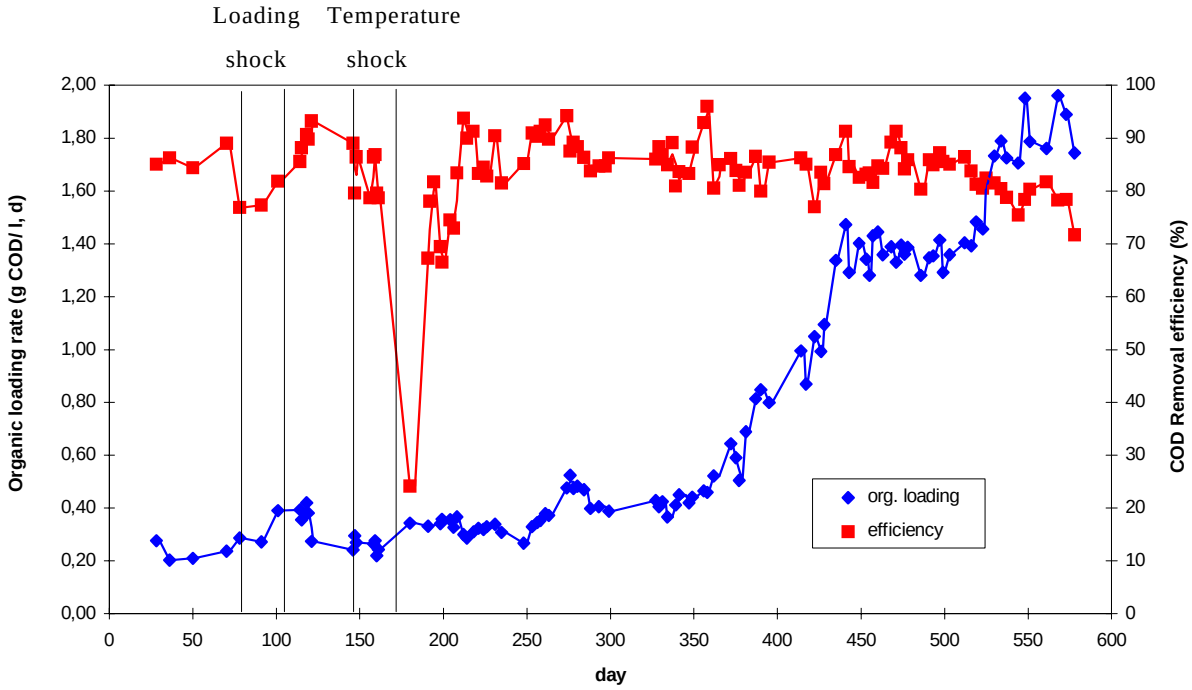


Fig. 3.11 c. Performance of reactor R 2 : COD load and % COD removal.

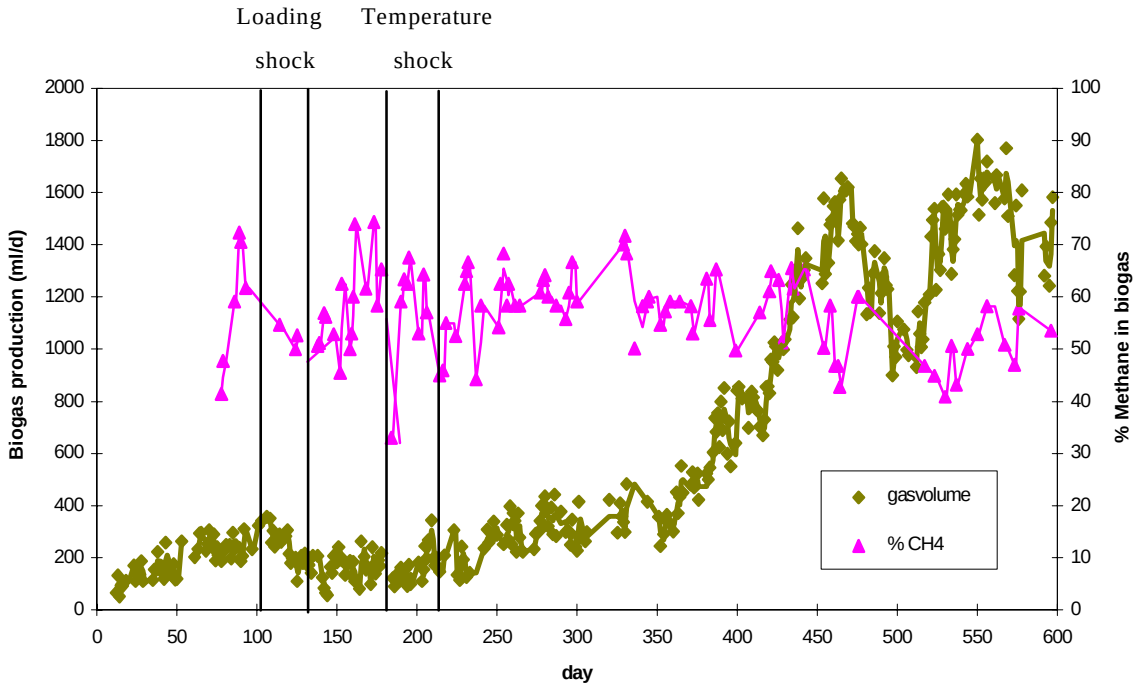


Fig. 3.11 d. Performance of reactor R 2 : Total gas production and % methane in biogas.

From start up until day 200 where the 2-CP loading rate was ≤ 50 mg/l, d, most of the biogas produced came from the degradation of glucose. After the system had recovered from the temperature shock, the total gas production increased along with the increasing glucose and 2-CP loading rate and reached 1.6 l/d on day 470 (Fig. 3.11 d). During days 470-500, biogas production decreased from 1.6 to 1.0 l/d and then increased again to

the preceding level on day 530. A decrease of total gas production can be seen again at the end of the experiment where the 2-CP loading rate was over 600 mg/l, d. A biogas production of about 200-300 ml less than the theoretically expected value (biogas production from glucose + 2-CP) at each loading rate was observed, presumably due to incomplete degradation of 2-CP to CH_4 and CO_2. The proportion of methane in the biogas, was between 50 and 70%. A lower methane content than 50% indicated a disturbance of methanogenesis in the reactor.

3.4.4 Chloride ion production

The net chloride production in the reactor was measured beginning with day 190 (after recovery from the temperature shock) to confirm the dechlorination of 2-CP. The results are presented in Figure 3.12. The maximum possible chloride ion production (drawn as a solid line in Figure 3.12) was calculated from the difference between the 2-CP concentration in influent and effluent, assuming reductive dechlorination with chloride ion release. A corresponding amount of chloride ions in the effluent to the theoretically expected maximum value was observed throughout the experiment, indicating that the disappearance of 2-CP in the reactor could be attributed to reductive dechlorination. At the termination of the experiment, the chloride concentration in the effluent had reached 350 mg/d which corresponded to 9.86 mM/d or 1267 mg/d of 2-CP that was dechlorinated.

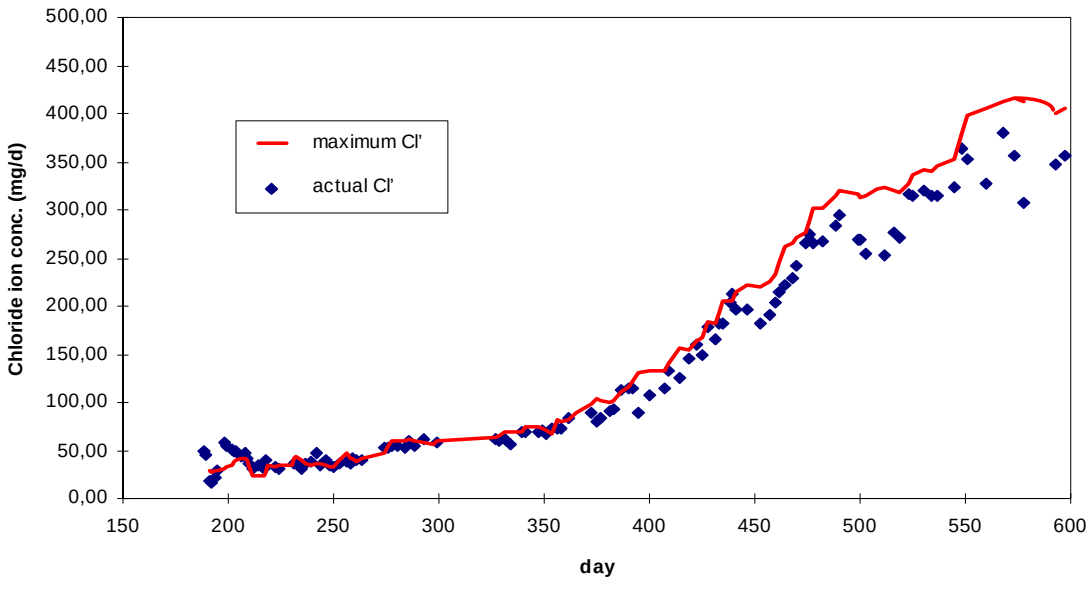


Fig 3.12 Actual and maximum net chloride ions production from the transformation of 2-CP in reactor R 2.

3.5 Mineralization and dechlorination rate of 2-CP in batch culture

Granular sludge from reactor R 2 (taken at day 90 after reactor start up) was incubated in serum bottles in the presence of 1 mM 2-CP +/- 5 mM glucose as a co-substrate. The initial dechlorination rate of glucose-amended cultures was about 43 mg/l,·d and phenol, the primary product from reductive dechlorination, was degraded within 4-5 days. When the 2-CP was completely mineralized, the same amount of 2-CP +/- glucose was refeeded. After 4 times of refeeding, the dechlorination rate of glucose-amended cultures had improved to approximately 64 mg/l,·d but the removal of phenol still required the same time (Fig. 3.13 a). The addition of 5 mM glucose after 2-CP was completely dechlorinated had no significant effect on the phenol degradation rate of this culture.

The initial dechlorination rate of non-glucose amended cultures was only 22 mg/l,·d but the phenol removal rate was the same as in glucose-amended cultures (Fig. 3.13 b). Refeeding

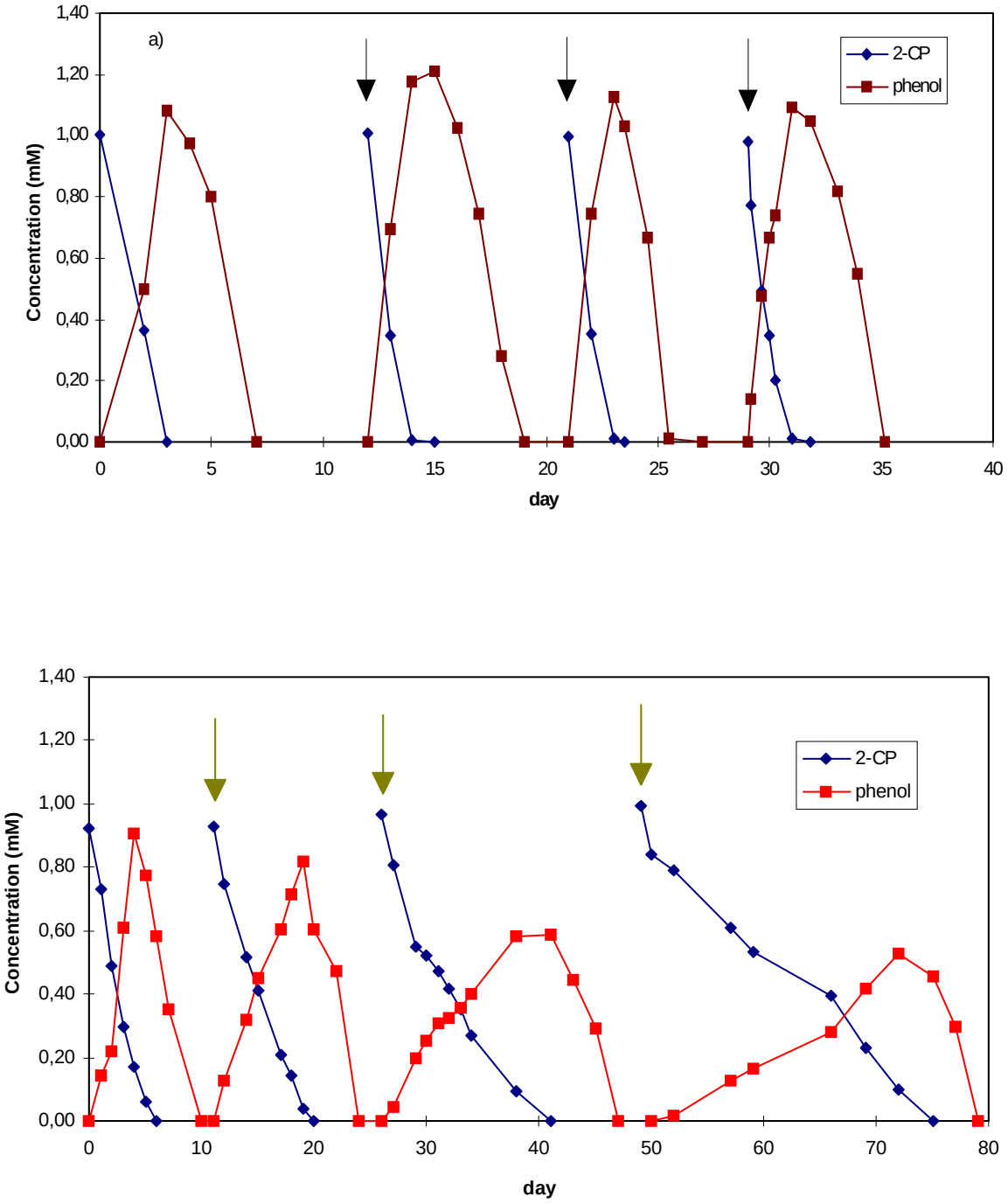


Fig. 3.13 Degradation of 2-CP and phenol in the cultures amended with glucose as co-substrate (a) and in non-glucose amended cultures (b).

of 2-CP could improve neither dechlorination nor the mineralization rate of this culture. On the other hand, the dechlorination rate was gradually decreasing from 22 mg/l,·d (1st addition) to 5 mg/l,·d (4 th addition). Whereas 1 mM 2-CP was completely degraded to methane and CO_2 in the glucose-amended batch cultures within 5-7 days in the non-glucose-amended cultures complete degradation required 10-20 days.

Theoretically 1 mM of 2-CP was dechlorinated to 1 mM of phenol (Eq. 2) and when 2-CP finally was completely mineralized, 3.25 mM of CH_4 should be obtained (Eq. 3).

$$C_6H_4OHCl + H_2 \longrightarrow C_6H_5OH + HCl \quad \text{.....(2)}$$

$$C_6H_4OHCl + 4.5\ H_2O \longrightarrow 2.75\ CO_2 + 3.25\ CH_4 + HCl \quad \text{.....(3)}$$

The amount of methane in the first 9 days of the experiment, in which only 2-CP in glucose-amended cultures was completely degraded, corresponded to the stoichiometric value expected for complete degradation of 2-CP (Fig. 3.14). The formation of methane in both cultures indicated that dechlorination did not inhibit methanogenesis from glucose.

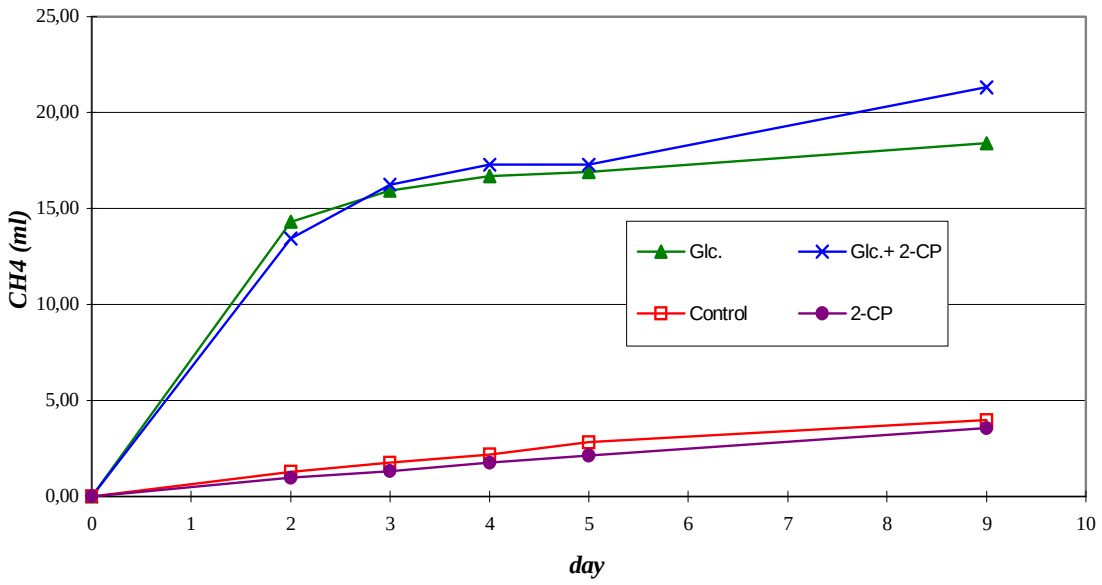


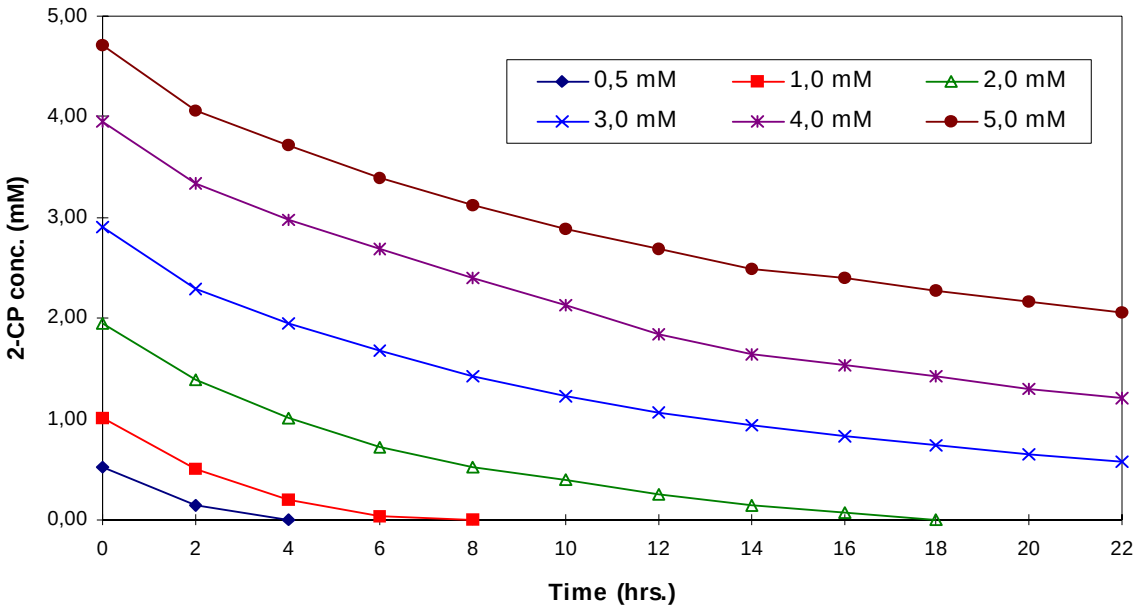
Fig. 3.14 Methane produced in glucose-amended cultures and non-glucose amended cultures in the presence of 2-CP. Control = background methane formation of this culture

3.6 Dechlorination kinetics

2-CP degrading granules were taken from reactor R 2 at a 2-CP loading of between 51 and 116 mg/l, d (day 220-360 of reactor operation) to determine the specific dechlorination rate and kinetic parameters of the dechlorination. Concentrations of 2-CP used were between 0.5-5 mM and a fixed glucose concentration of 0.9 g/l (5 mM) served as co-substrate in each sample. The depletion curve of 2-CP and the amount of phenol, the first intermediate of the dechlorination, from one of the experiments are demonstrated in Figure 3.15 and 3.16. It can be seen that the dechlorination rate was drastically increased (7-9 times) compared to the rate at the start of the reactor (see 3.5). This suggests a good adaptation of this biomass to 2-CP.

Fig. 3.15 Biotransformation of different concentrations of 2-CP.

Figure 3.17 shows the results of the kinetic experiment. The dechlorination rates were calculated as the



slope of the regression line of the 2-CP depletion curve. Each initial concentration of 2-CP was the actual concentration measured at the begining of the experiment (t = 0). Determination of the half velocity constant (K_m)and the maximum dechlorination rate (V_{max}) using a graphical method according to Lineweaver - Burk gave a K_m value of 1.18 mM (= 152 mg/l) and a V_{max} of 66.4 mg/g TS, d.

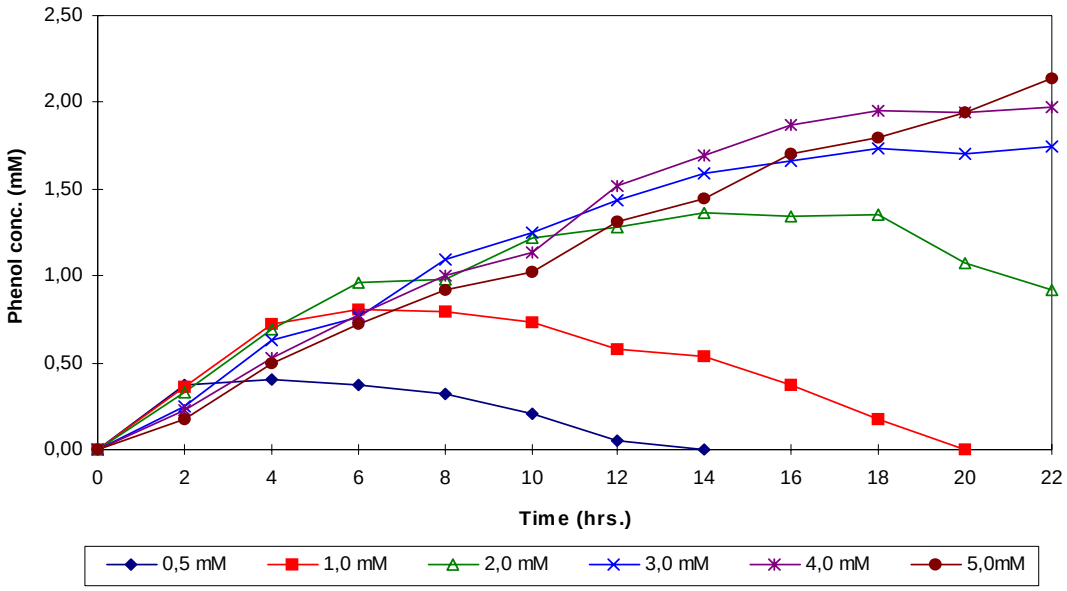


Fig. 3.16 Amount of phenol that occured during dechlorination of various concentrations of 2-CP.

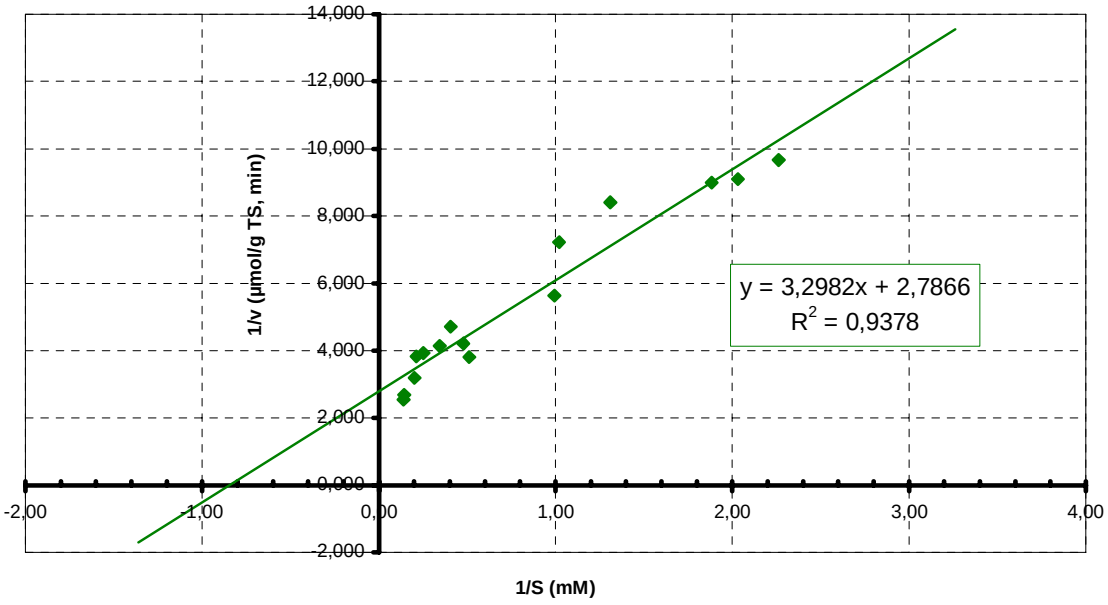


Fig. 3.17 Lineweaver-Burk plot to determine the maximum dechlorination rate V_{max} and the half velocity constant, K_m.

With respect to V_{max} , a 2-CP loading rate of 511 mg/l, d (based on a total biomass concentration of 20 g) was estimated as the maximum dechlorination rate for this system. Therefore, the 2-CP loading rate of reactor R 2 was stepwise increased by decreasing the HRT or increasing the 2-CP concentration in the influent (starting

from day 360 on). As shown in Figure 3.11 b., a 2-CP loading rate of more than 600 mg/l, d in the last phase of operation verified the validity of this estimation.

On day 462 of the reactor operation, biomass was taken to determine the specific dechlorination rate again. It was found that the initial dechlorination rate of 2-CP for a concentration range from 0.5-5 mM 2-CP in each sample was in the same range between 0.07-0.08 mM/h (Fig. 3.18).

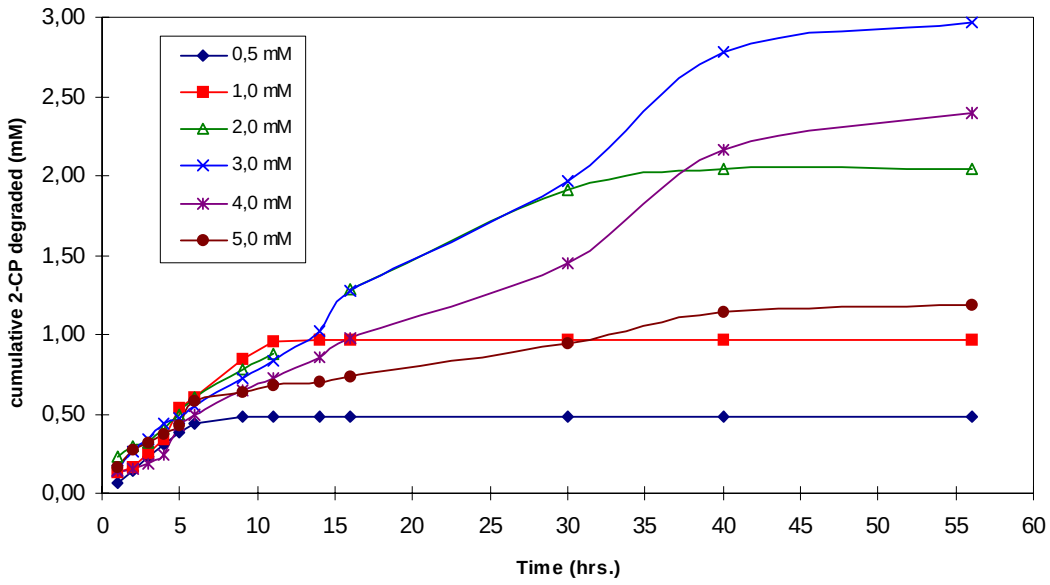


Fig. 3.18 Dechlorination of 2-CP at various initial concentrations of 2-CP.

3.7 Effect of glucose on dechlorination at higher 2-CP concentration

Reactor R 2 was operated for more than 350 days with increasing 2-CP concentration. A chlorophenol removal efficiency over 95 % was attained at a fixed glucose concentration of 0.9 g/l. This indicated the potential ability of these sludge granules to use 2-CP as another C-source. Moreover, it can be seen from Figure 3.15 that 2-CP in samples which have concentrations ≤ 2 mM was removed within 24 hours in the presence of 5 mM glucose. Thus, it should be examined whether glucose still enhanced the dechlorination by this biomass at 2-CP concentration higher than 2 mM.

The 2-CP degrading granules were incubated with 3, 4 and 5 mM 2-CP in the presence/absence of 5 mM glucose. The repeated addition of the same concentrations of glucose was performed in one of the glucose-amended samples when the initial amount of glucose was exhausted. The concentration of 2-CP and phenol in each sample is shown in Figure 3.19 (a-c). The acceleration of dechlorination by repeating glucose additions was markedly seen only in samples which were incubated with 4 mM of 2-CP (Fig. 3.19 b). In these samples, 2-CP

was almost completely dechlorinated in 80 hours while it took more than 120 hours in one-time glucose amended and non-amended samples. The dechlorination and phenol transformation rates of glucose-amended and non-glucose amended samples for 2-CP concentrations of 3 mM and 5 mM were not significantly different. Furthermore, repeated addition of glucose neither did enhance the dechlorination rate nor the phenol transformation rate, as can be obviously seen in samples incubated with 5 mM of 2-CP (Fig. 3.19 b). Despite 6 times of glucose refeeding within 5 weeks, the same amount of 2-CP (ca. 2 mM) was dechlorinated like in others samples. Results showed that glucose was still required by the adapted biomass for dechlorination, although no marked effect of glucose addition was observed in samples incubated with 3 and 5 mM of 2-CP. Therefore, 5 mM of glucose was further fed as co-substrate in the influent for reactor R 2 in order to ensure and enhance the dechlorination of 2-CP in the system.

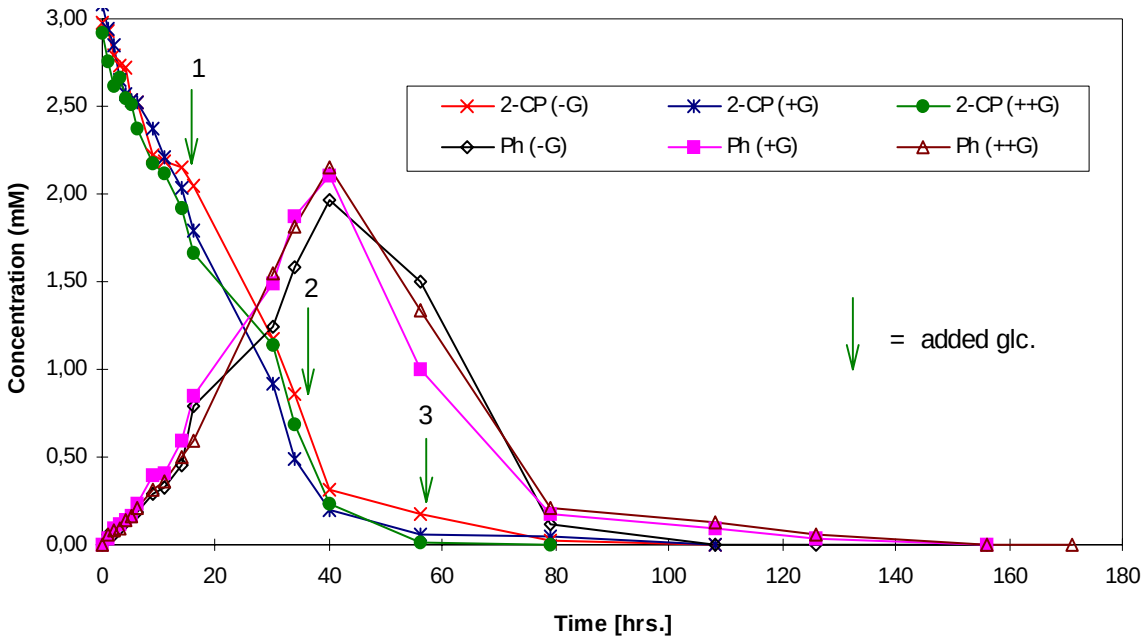


Fig. 3.19 a. 2-CP and phenol concentration in samples which were incubated with 3 mM of 2-CP an a different regime of glucose addition. Abbreviation : -G = no glucose addition, +G = one-time glucose addition, ++G = more times glucose addition.

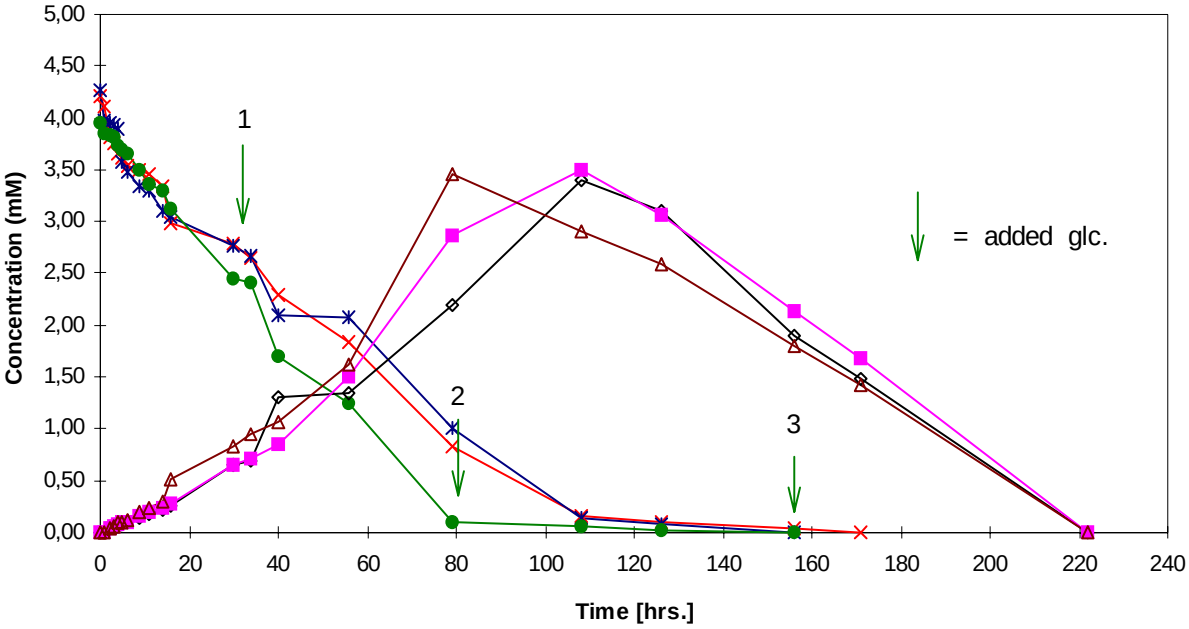


Fig. 3.19 b. 2-CP and phenol concentration in samples which were incubated with 4 mM of 2-CP and a different regime of glucose addition. Abbreviations and symbols are the same as in Fig. 4.19 a.

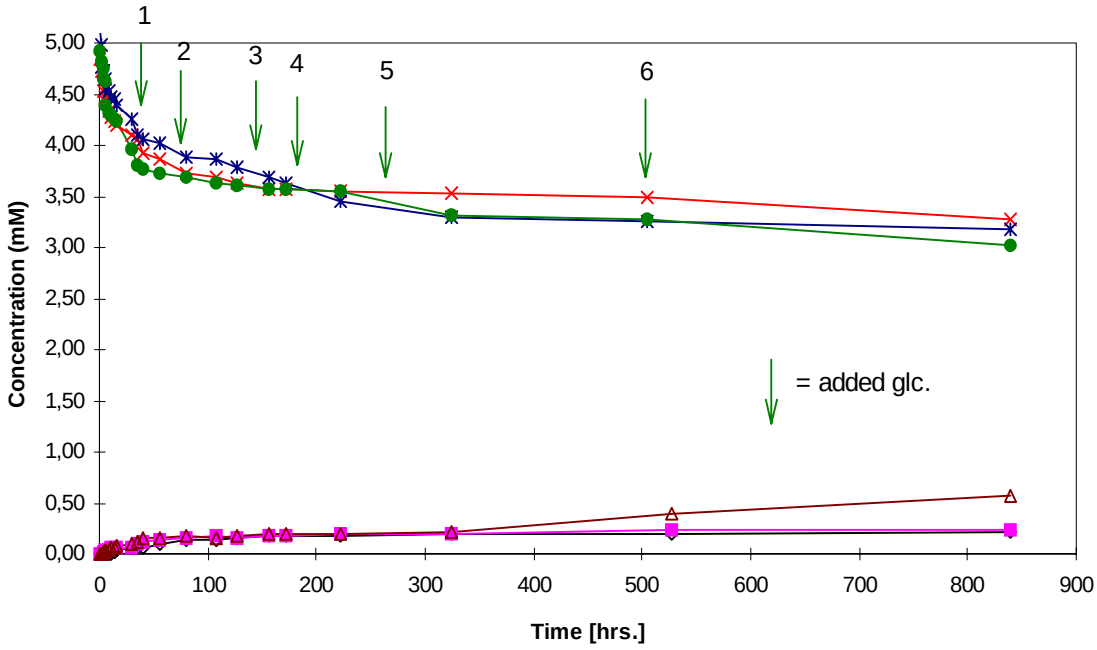
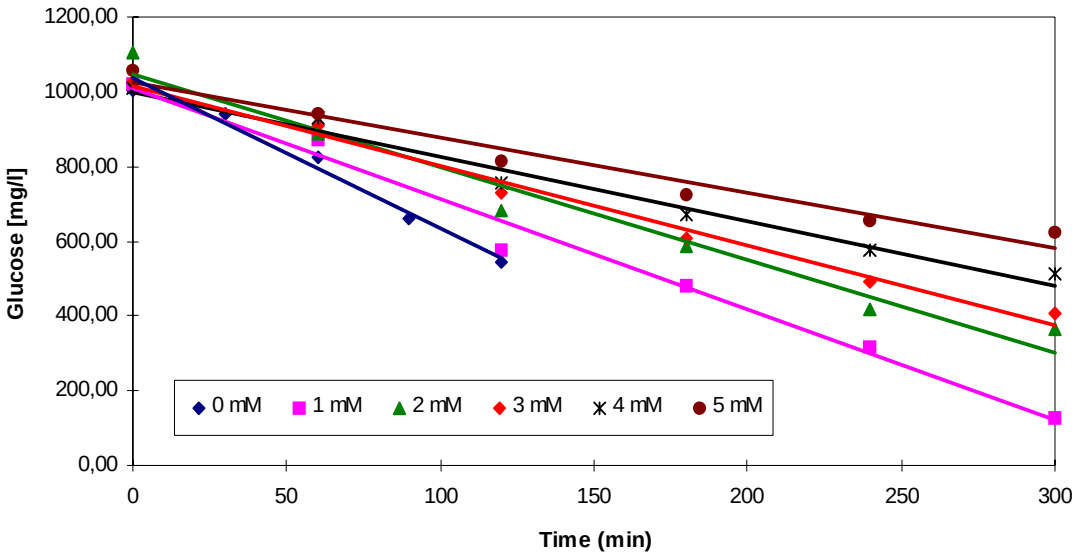


Fig. 3.19 c. 2-CP and phenol concentration in samples which were incubated with 5 mM of 2-CP and a different regime of glucose addition. Abbreviations and symbols are the same as in Fig. 4.19 a.

3.8 Kinetics of glucose degradation

Glucose was used as a co-substrate for treatment of a 2-CP containing synthetic wastewater in a UASB reactor to ensure the dechlorination evtl. degradation of 2-CP. Hence, the impact of 2-CP on kinetic parameters of glucose degradation by 2-CP degrading granules (adapted biomass) in comparison with glucose-degrading granules (non-adapted biomass) should be investigated.

Figure 3.20 illustrates the inhibition effect of 2-CP on glucose degradation of non-adapted biomass at a glucose concentration of 1000 mg/l. The glucose degradation rate (calculated from the slope of each curve) decreased with an increasing concentration of 2-CP. Figures 3.21 and 3.22 show the Dixon plot (a) and Cornish-Bowden plot (b) in the presence of different fixed glucose concentrations of non-adapted biomass and of biomass



that was adapted to 2-CP, respectively.

Fig. 3.20 Degradation of 1000 mg/l glucose by non-adapted (unacclimated) biomass which was incubated with various concentrations of 2-CP.

From the Dixon and Cornish-Bowden plot, it was concluded that 2-CP was an inhibitor of glucose degradation for both adapted and non-adapted biomass. The type of inhibition was a mixed inhibition in which for adapted biomass the K_i value (dissociation constant of the EI complex) was higher than K_{ii} (dissociation constant of the ESI complex) and vice versa for non-adapted biomass. The kinetic parameters for glucose degradation, estimated by the graphical method, are presented in Table 3.2.

Table 3.2 : Kinetic parameters for glucose degradation by non-adapted and adapted biomass in the presence of 2-CP concentrations ranging from 0 to 5 mM.

kinetic parameters	**V_{max}** (g glc./gTS, d)	**K_m** (mg glc./l)	**K_i** (mg 2-CP/l)	**K_{ii}** (mg 2-CP/l)
non-adapted biomass	1.1486	22.21	116.27	501.32
adapted biomass	2.0065	322.07	705.72	152.37

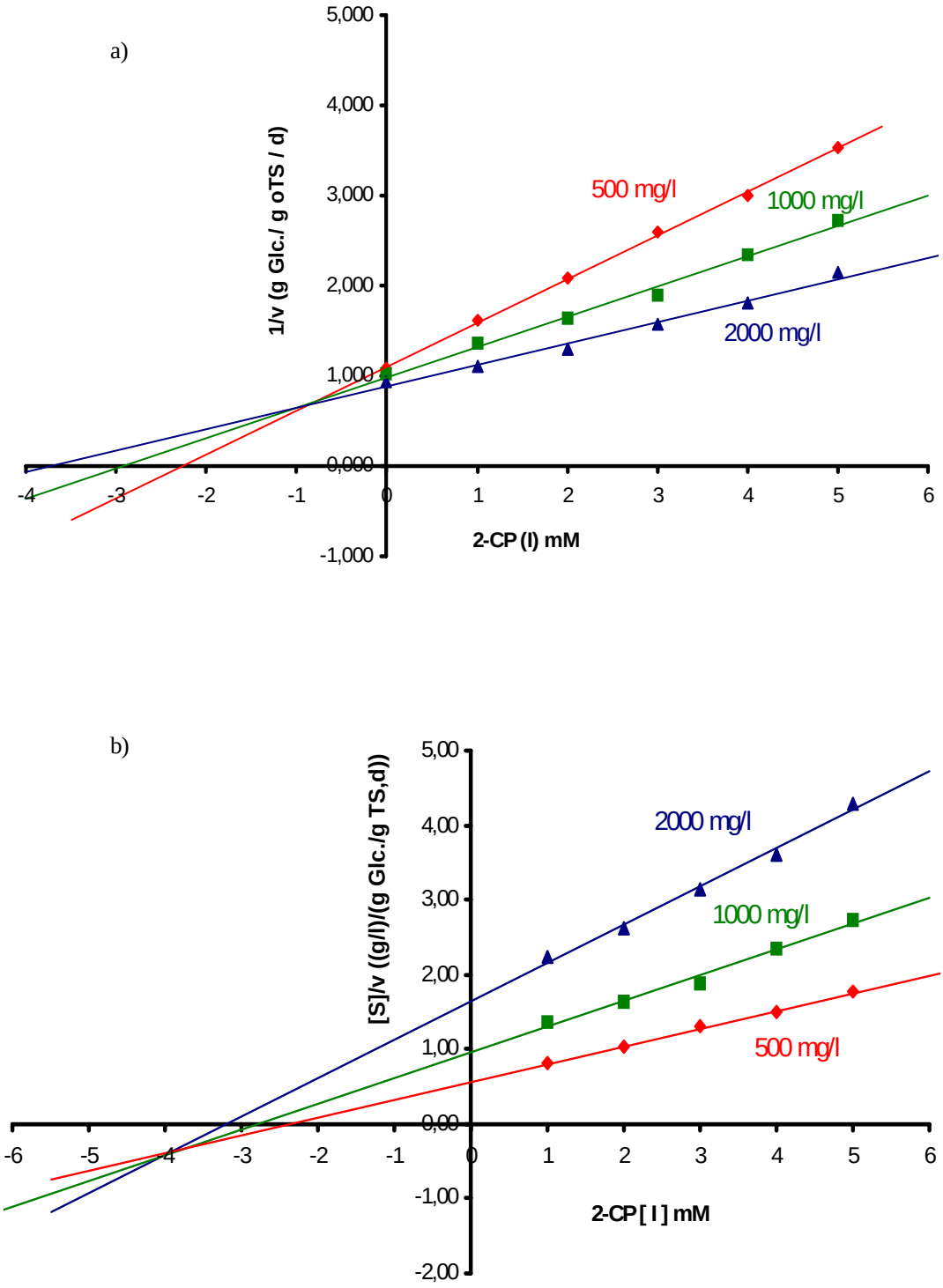


Fig. 3.21 Dixon (a) and Cornish-Bowden plot (b) of non-adapted biomass in the presence of 5 different concentrations of 2-CP and three different concentrations of glucose.

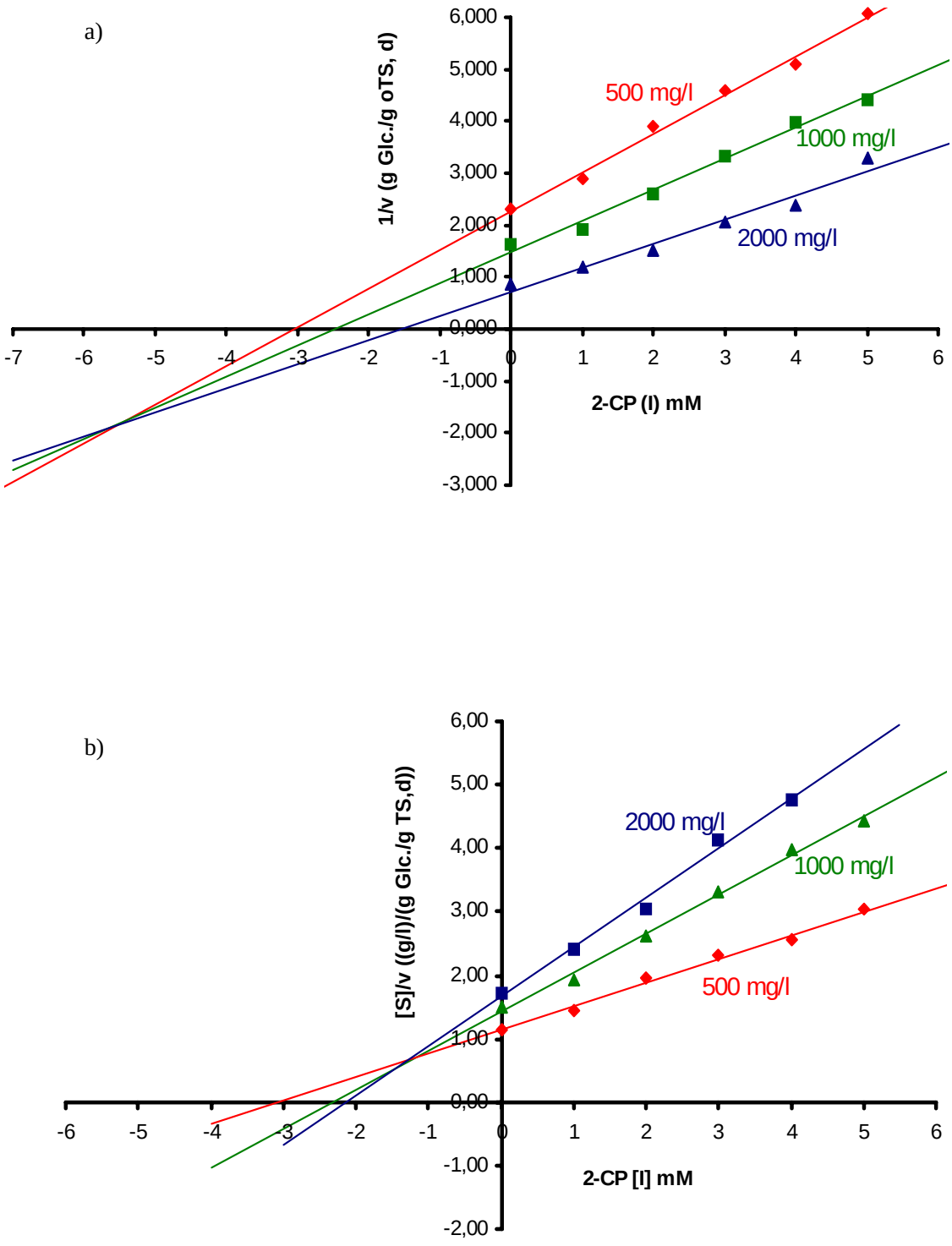


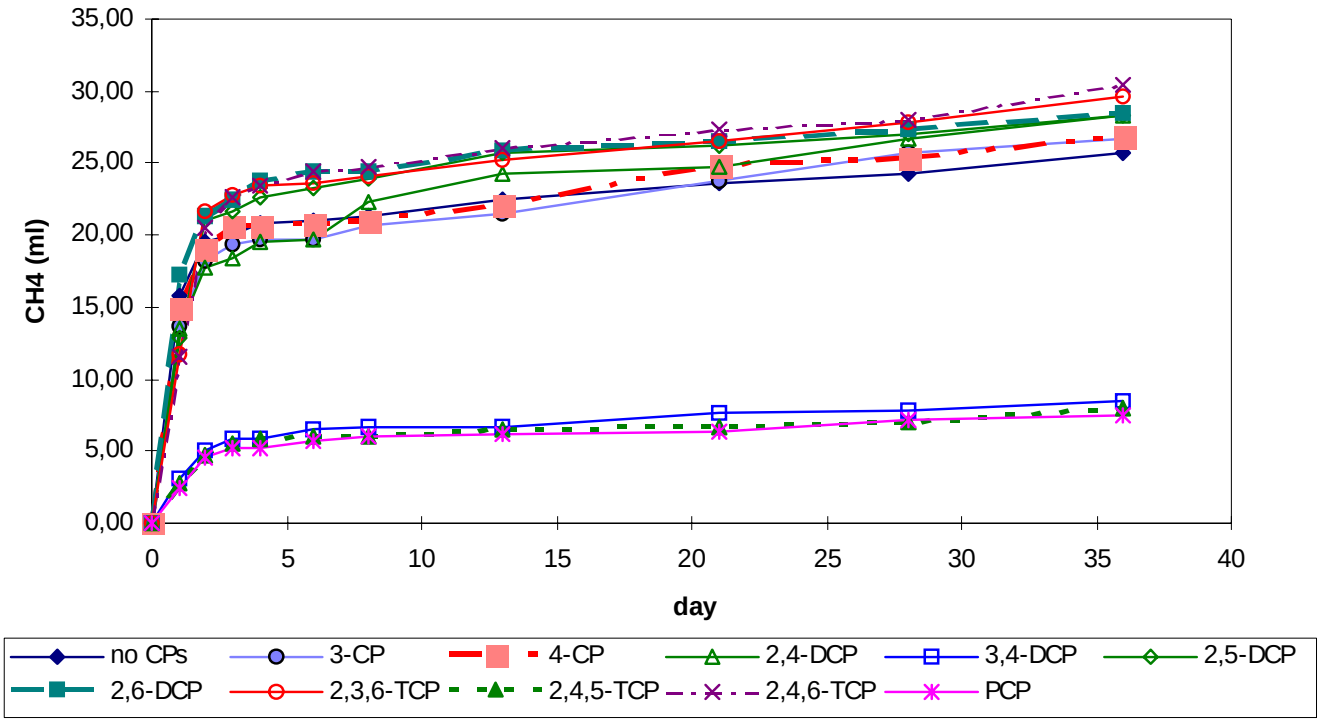
Fig. 3.22 Dixon (a) and Cornish-Bowden plot (b) of adapted biomass in the presence of 5 different concentrations of (2-CP) and three different concentrations of glucose.

3.9 Degradation potential of 2-CP-degrading granules for other chlorophenols

3.9.1 Glucose as electron donor

The 2-CP degrading granules from reactor R 2 were incubated with 0.5 mM of 10 different chlorophenols (3-CP, 4-CP, 2,4-DCP, 2,5-DCP, 2,6-DCP, 3,4-DCP, 2,3,6-TCP, 2,4,5-TCP, 2,4,6-TCP, and PCP) and 5 mM of glucose. The amount of methane gas produced in each sample over 35 days of incubation is shown in Figure 3.23. Figure 3.24-3.26 show the concentration of parental mono-, di-, tri- or pentachlorosubstituted phenols evtl. the intermediates and the net chloride ion concentration in each sample.

Fig. 3.23 Amount of methane produced in the samples which were incubated with different chlorophenols



The 2-CP degrading granules were able to completely degrade 4-CP within 13 days whereas the transformation of 3-CP took longer time with a little initial lag period (Fig. 3.24). The amount of chloride ions released from transformation of both MCPs corresponded with the quantity of biotransformed chlorophenols. No phenol was detected during transformation of 3-CP and 4-CP by this culture, indicating that phenol was not the rate-limiting step and presumably was further degraded to methane and CO_2 .

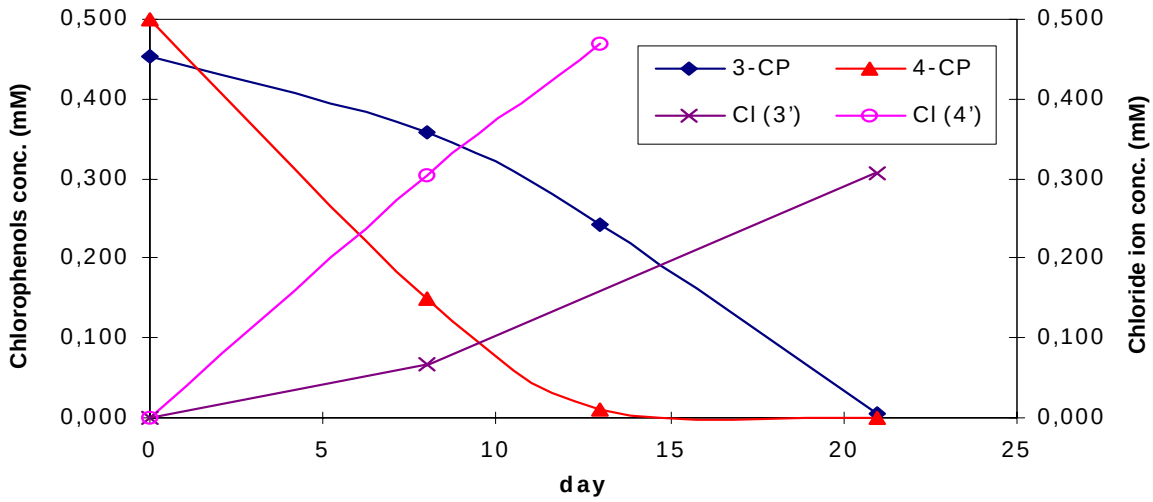


Fig. 3.24 Degradation of 3-CP, 4-CP and amount of chloride ions released during dechlorination by 2-CP degrading cultures.

Except for 3,4-DCP all tested dichlorophenols were completely degraded in a period of time, ranging from less than 2 days to 40 days (Fig. 3.25). The transformation of 3,4-DCP occured mainly in the first few days of incubation but about half of the initial concentration remained in the sample. Two monochlorophenols, 3-CP and 4-CP, were found as intermediates from the dechlorination of 3,4-DCP but in little less than stoichiometric amounts (Fig. 3.25 b). 2,4-DCP and 2,5-DCP were first dechlorinated to 4-CP and 3-CP, and were then completely transformed in 15 and 36 days, respectively (Fig 3.25 a, c). Total disappearance of 2,6-DCP occured in less than 2 days and no other chlorophenols or phenol was detected in this sample (Fig. 3.25 d). An amount of chloride ions corresponding to the amount of transformed dichlorophenol was found in each tested sample.

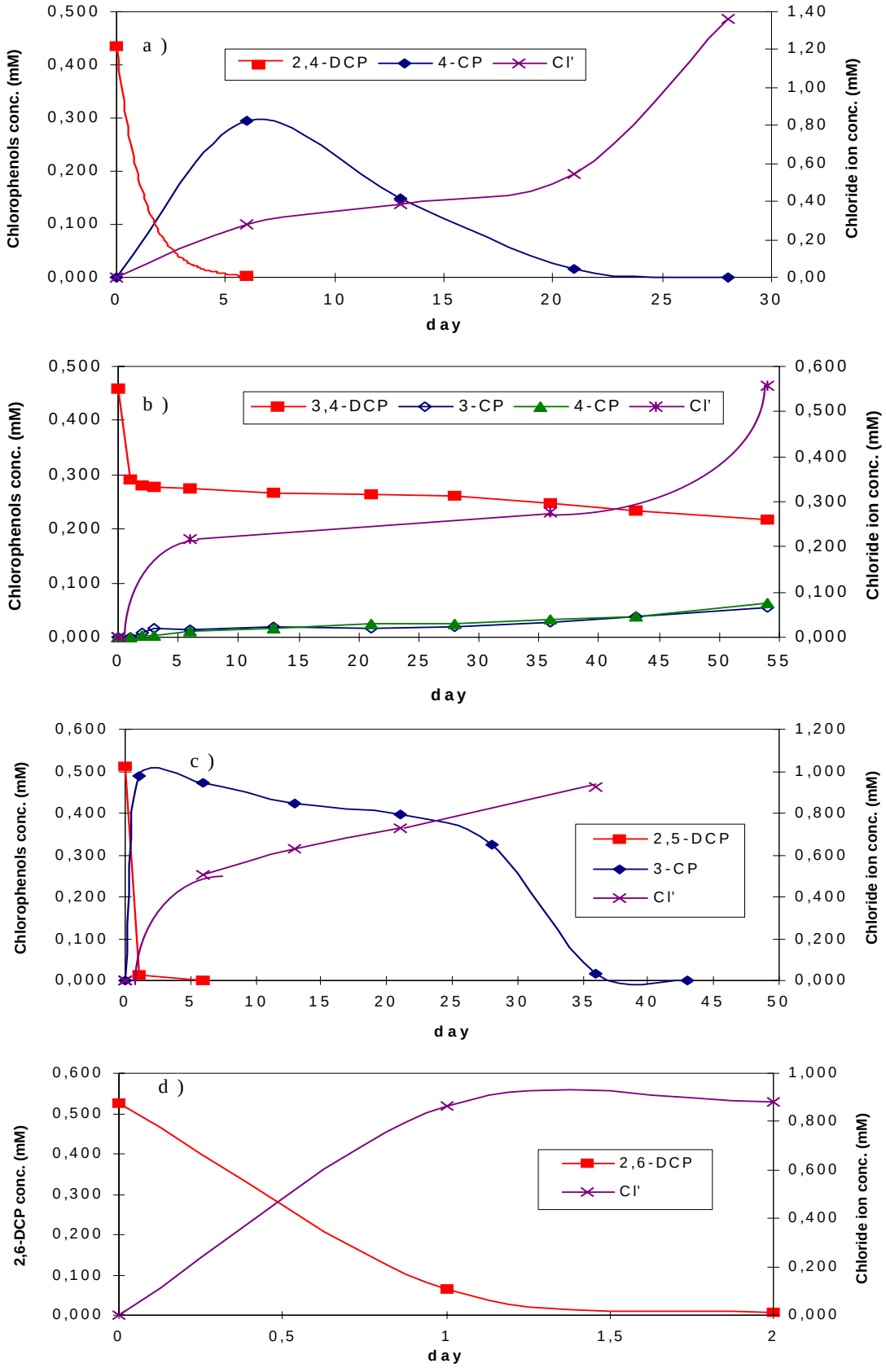


Fig. 3.25 Degradation of (a) 2,4-DCP (b) 3,4-DCP (c) 2,5-DCP (d) 2,6-DCP and amount of chloride ions released from the dechlorination by 2-CP degrading cultures.

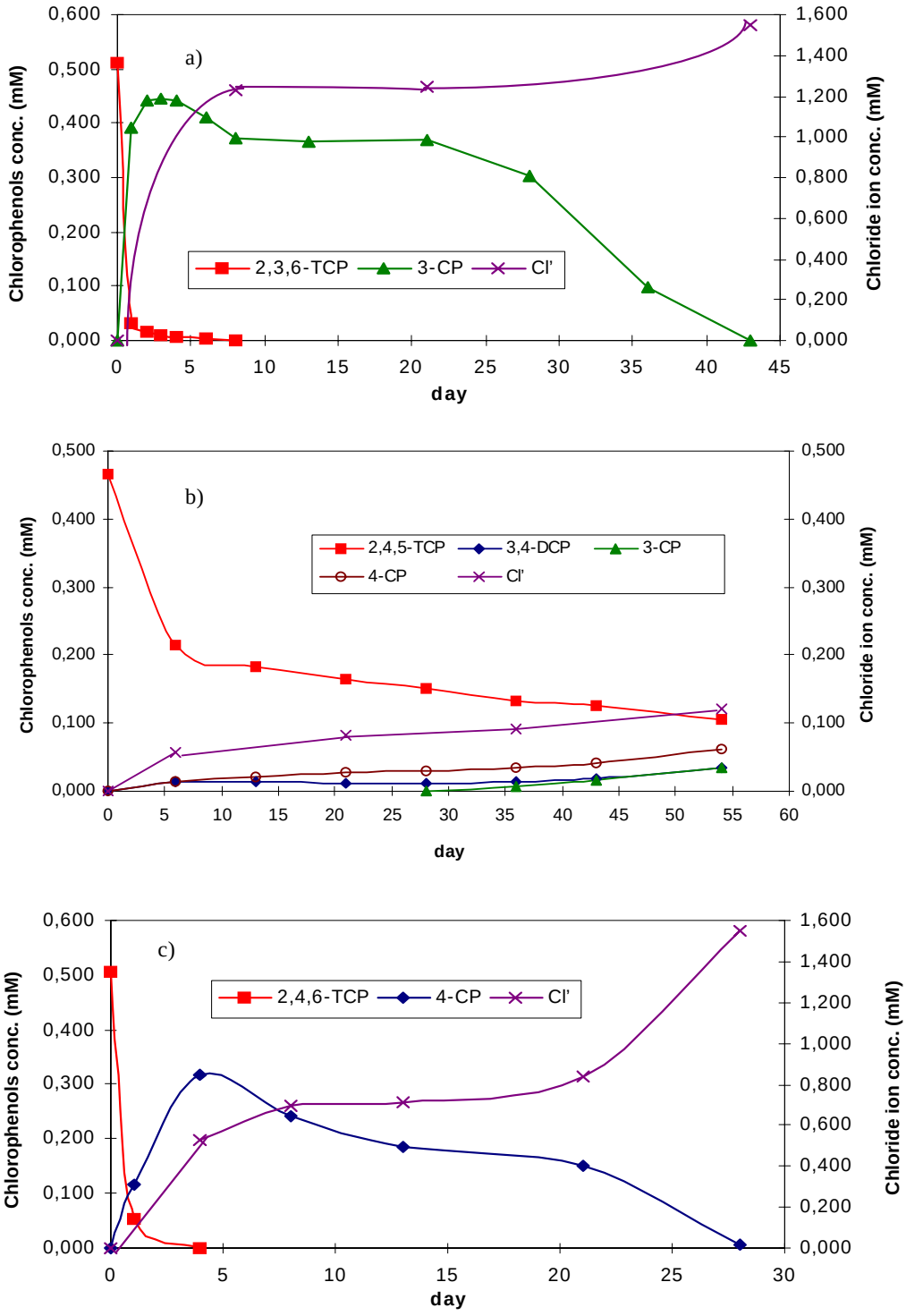


Fig. 3.26 Degradation of (a) 2,3,6-TCP (b) 2,4,5-TCP (c) 2,4,6-TCP and amount of chloride ions released from the dechlorination by 2-CP degrading cultures.

The complete transformation of trichlorophenol by 2-CP degrading granules was observed in samples which were incubated with 2,3,6-TCP and 2,4,6-TCP. Both trichlorophenols were double dechlorinated to 3-CP and 4-CP, respectively, without the appearance of any DCP as intermediate (Fig 3.26 a, c). Moreover, the stoichiometric amount of chloride ions in both samples was also obtained. The sequential dechlorination was observed from the transformation of 2,4,5-TCP, in which 2,4,5-TCP was dechlorinated via 3,4-DCP to 3-CP and 4-CP (Fig. 3.26 b). However, the amount of intermediates was relatively low compared to amount of 2,4,5-TCP that disappeared.

Pentachlorophenol was mainly removed at the begining of the incubation by adsorption. After that the concentration of PCP was nearly unchanged (Fig. 3.27). Some TCP, DCP, MCP and phenol were found in the samples which were incubated with PCP, indicating that there was little biotransformation and not only adsorption to the sludge pellets. Due to the lack of some TCP references and due to the fact that all DCP have nearly the same retention time under the analytic conditions, the peak area of each substance was presented instead of a concentration to demonstrate the occurence of each intermediate in the samples (Figure 3.28). Trichlorophenol (2,4,5-TCP and an unknown isomer) was the major intermediate on day 13 but disappeared after that. On day 28, only MCP and phenol were observed in the samples. TCP (2,4,5- and 2,4,6-TCP) appeared again on day 43 while DCP was first detected on day 54 of the incubation. 3-CP, 4-CP and phenol were always found throughout the incubation period and had a trend to increase with the time.

Methane formation from glucose in samples which were incubated with 0.5 mM 3,4-DCP, 2,4,5-TCP and PCP was more than 80 % inhibited whereas in other samples the amount of methane corresponded to the amount of added glucose. Moreover, a higher methane amount than in control samples (granules incubated only with 5 mM glucose) was observed when the parent chlorophenols were completely removed (Fig. 3.23).

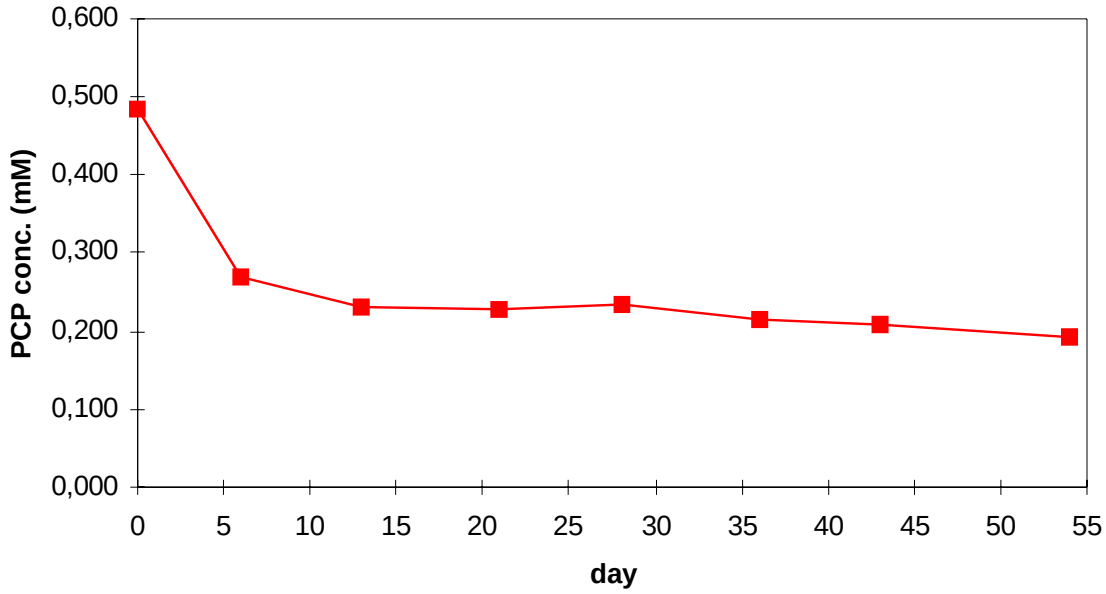


Fig. 3.27 Adsorption / transformation of PCP by 2-CP degrading cultures.

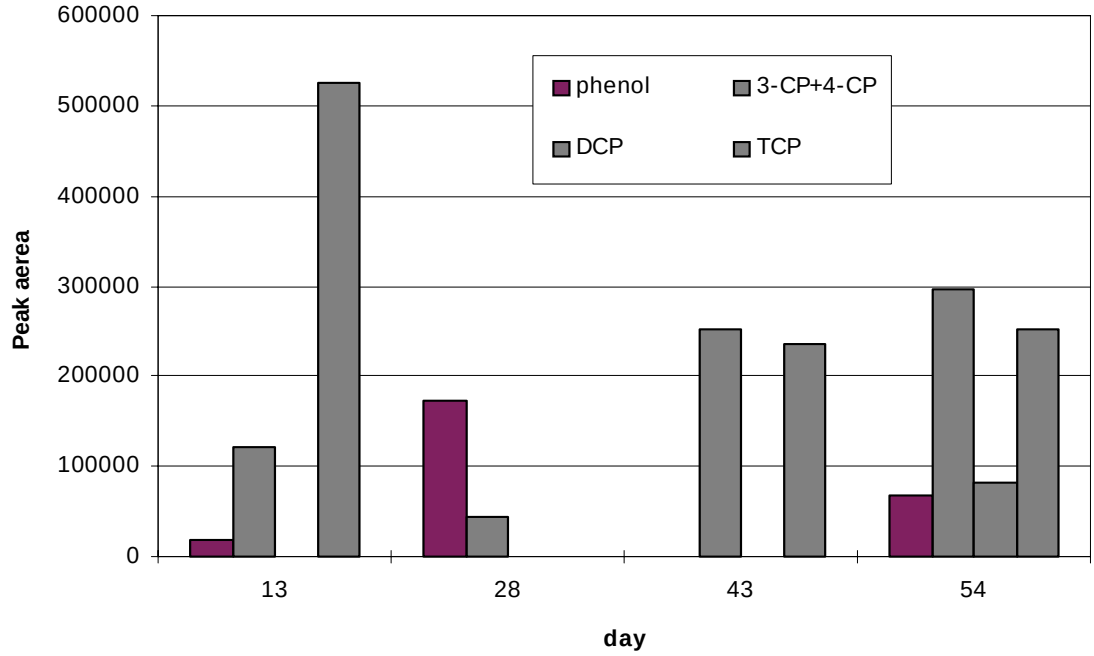
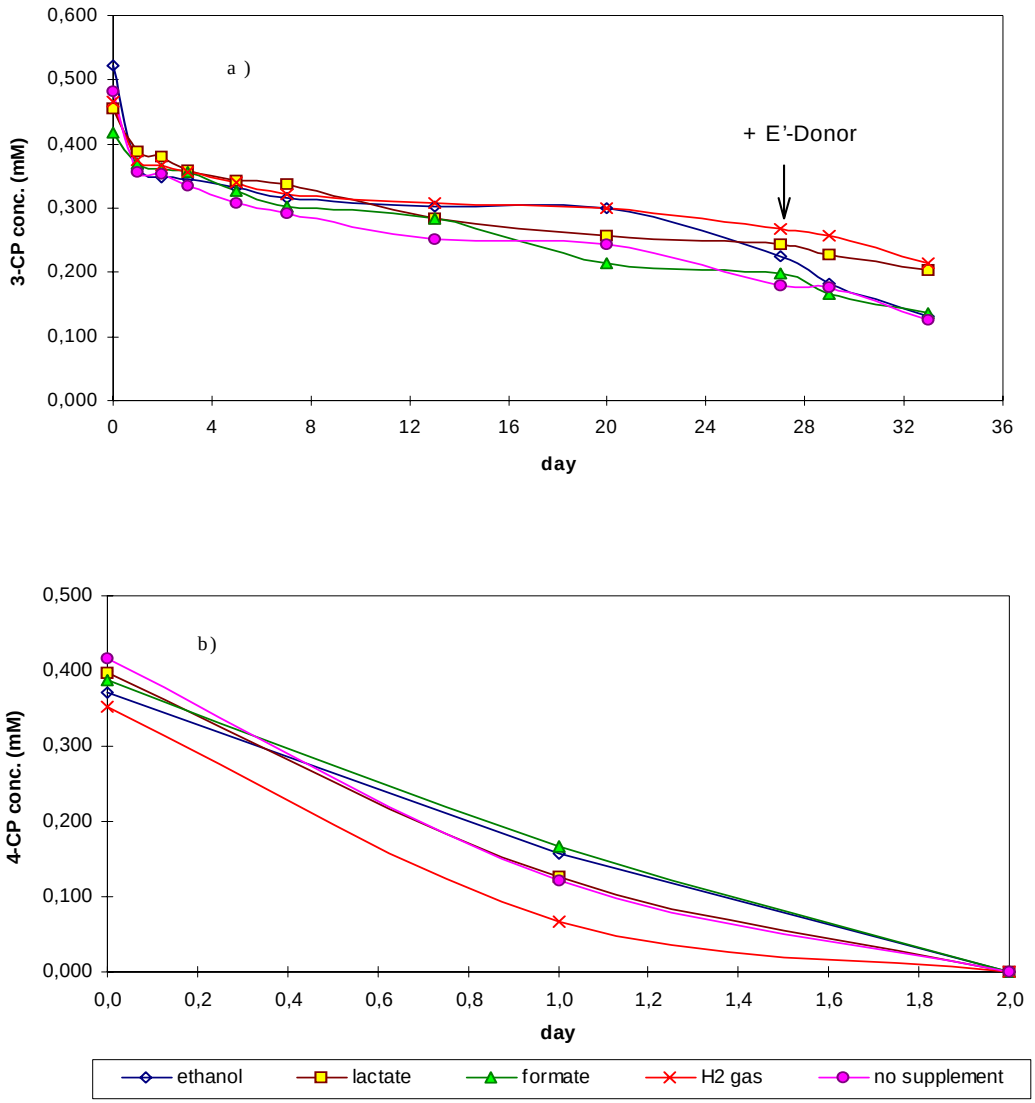


Fig. 3.28 Intermediates (expressed in form of peak area) occuring during the transformation of PCP by 2-CP-degrading cultures.

3.9.2 Other electron donors

Apart from glucose, other organic substrates (i.e. ethanol, lactate, formate) in concentration of 5 mM and 1 atm of H_2 gas were used as electron donors for evaluation of the potential of 2-CP degrading granules to transform other chlorophenols. A sample without any electron donor was also tested. Table 3.3 shows % transformation of each chlorophenol, incubated with various electron donors after 27 days of incubation.

Fig. 3.29 Transformation of 3-CP (a) and 4-CP (b) by 2-CP degrading cultures amended with various electron donors.



In the presence and absence of these electron donors, 3-CP was less transformed than when it was incubated with glucose (total dechlorination after 21 days) and it was not completely removed within 32 days of the incubation (Fig. 3.29 a). About 50 % of 3-CP was dechlorinated when ethanol, lactate, H_2 and formate were used as electron donors but little more 3-CP was transformed in non-amended cultures (see Table 3.3). In

contrast to 3-CP, total disappearance of 4-CP occured within 2 days with all electron donors and 4-CP was faster transformed when hydogen gas was used as electron donor (Fig. 3.29 b). Dechlorination of 2-CP and 4-CP of granules adapted to 2-CP occured at a similar rate, whereas 3-CP could only be dechlorinated at a much lower rate.

3,4-DCP was mainly removed at the begining of the incubation. Only 3-CP was found as the intermediate from the dechlorination of 3,4-DCP (Fig. 3.30 a, b), indicating that chlorine removal from the *meta*-position was much slower than from the *para*-position.

No complete degradation of 2,4-DCP, 2,5-DCP and 2,6-DCP was observed in this experiment. This may have been due to high initial concentrations of the parent substances (between 1.1-1.3 mM). The concentrations of these three dichlorophenols and of their intermediates are presented in Figure 3.31-3.33. The chlorine atom at the *ortho* position of 2,4-DCP, 2,5-DCP and 2,6-DCP was first dechlorinated and yielded 4-CP, 3-CP and 2-CP as intermediate, respectively. It was rather surprising that 2,6-DCP was the least transformed among these three DCPs, opposed to the rapid removal of this DCP isomers when incubated with glucose. In addition, 2-CP begun to accumulate in some samples after 8 days of incubation (Fig. 3.33 b). During 27 days of incubation, only the disappearance of 3-CP, an intermediate from 2,5-DCP transformation was observed (Fig. 3.32 b).

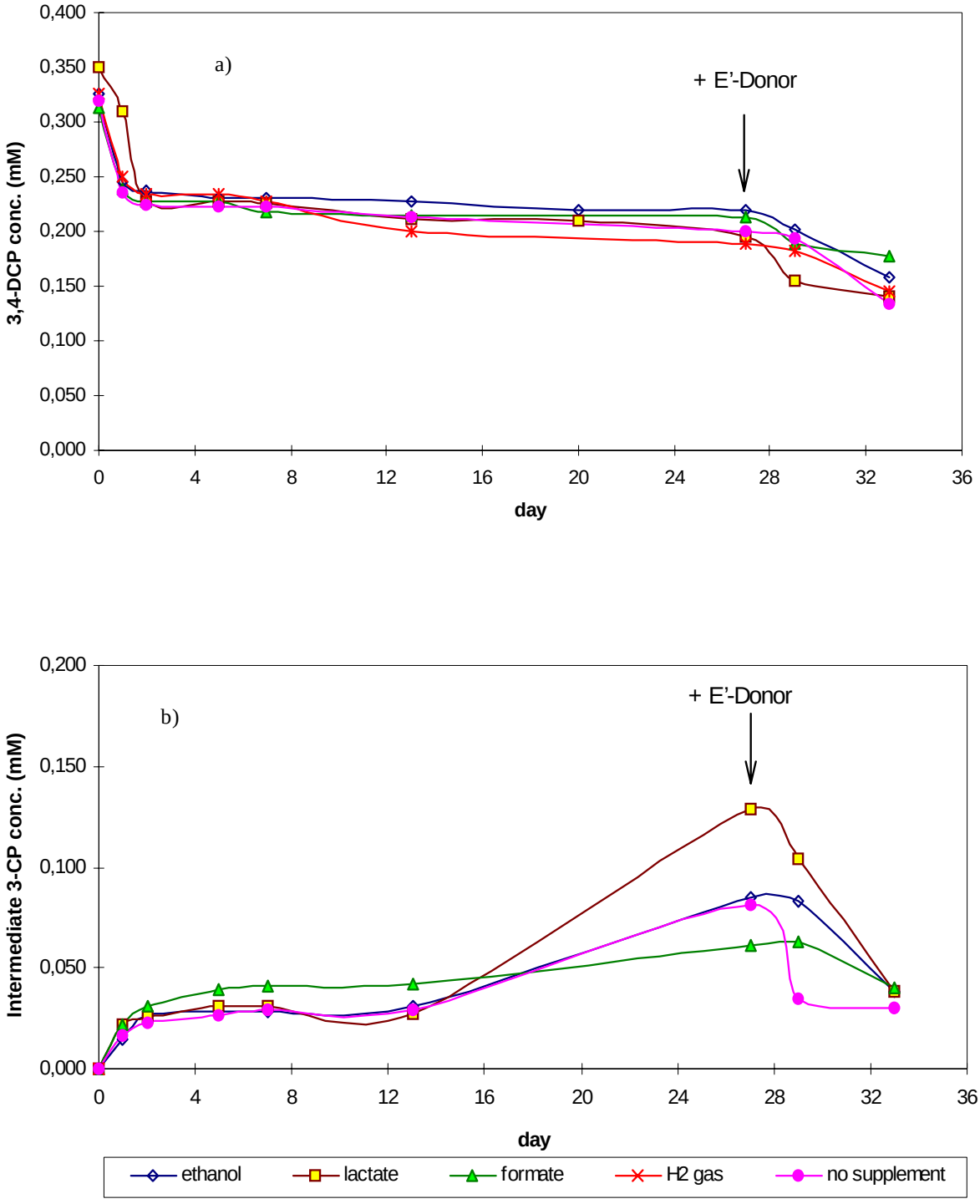


Fig. 3.30 Transformation of 3,4-CP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 3,4-DCP (b) concentration of 3-CP (intermediate from dechlorination of 3,4-DCP).

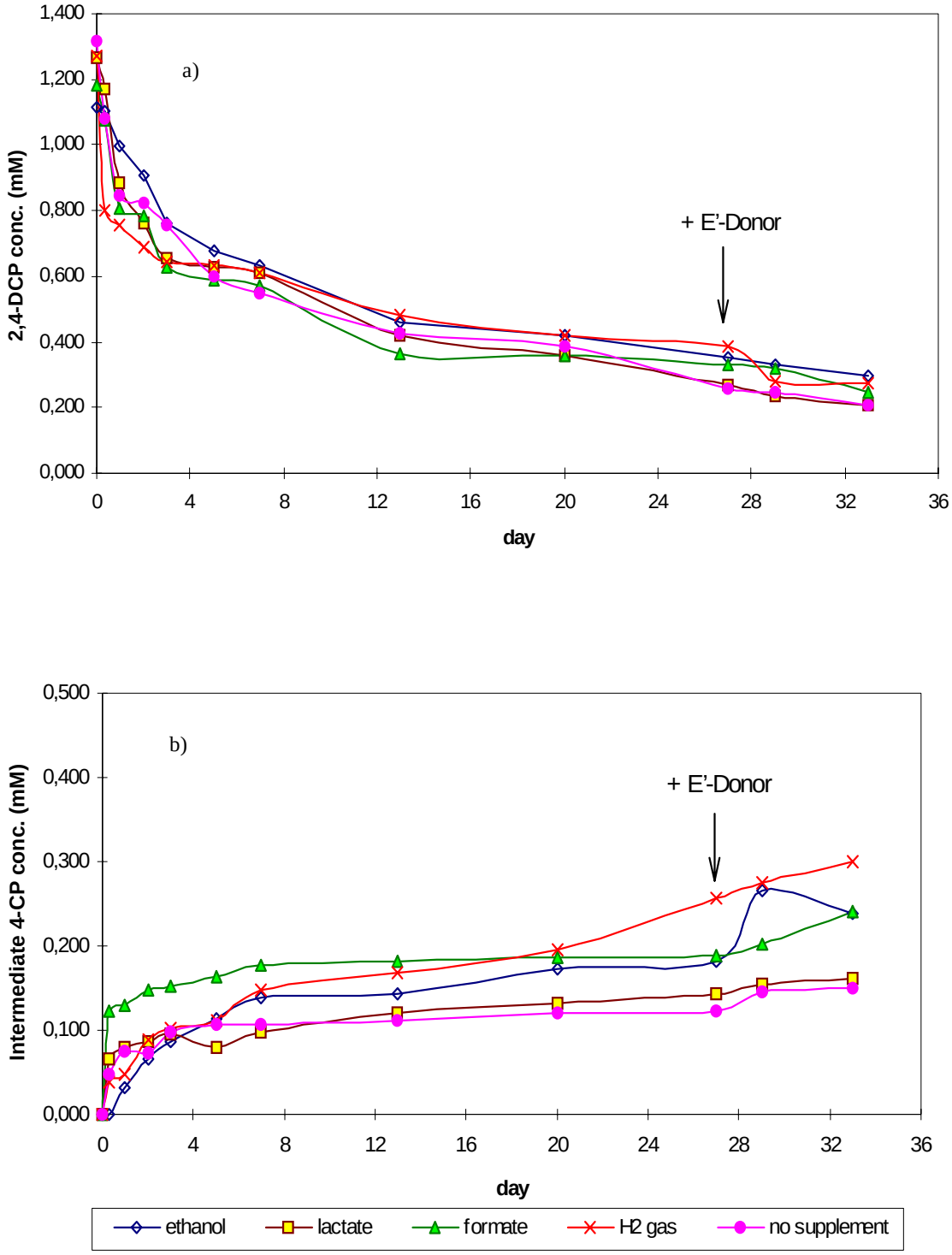


Fig.3.31 Transformation of 2,4-DCP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 2,4-DCP (b) concentration of 4-CP (intermediate from dechlorination of 2,4-DCP).

Table 3.3 Percentage transformation of chlorophenols incubated with various electron donors on day 27 of incubation.

Chlorophenol	% Transformation				
	ethanol	lactate	formate	H_2 gas	no supplement
3-CP	56.98	46.37	52.39	42.37	62.53
4-CP [1)]	57.68	68.59	56.96	81.30	70.91
2,4-DCP [2)]	68.22	78.70	72.23	69.76	80.44
2,5-DCP [2)]	61.05	70.28	66.91	74.70	65.42
2,6-DCP [2)]	38.71	35.71	33.97	56.27	31.71
3,4-DCP	32.62	44.13	39.94	43.69	39.50
2,3,6-TCP	47.78 [3)]	60.04 [3)]	53.01 [3)]	33.57 [3)]	60.40 [3)]
2,4,6-TCP	56.33 [4)]	64.29 [4)]	54.80 [4)]	49.16 [1)]	57.59 [4)]
2,4,5-TCP	77.89	72.95	71.51	64.12	73.67
PCP	60.38	52.31	56.73	45.45	54.18

Note : 1) % transformation after 1 day of incubation

2) initial concentrations are between 1.1-1.3 mM

3) % transformation of 3-CP, a product from reductive dechlorination of 2,3,6-TCP

4) % transformation of 4-CP, a product from reductive dechlorination of 2,4,6-TCP

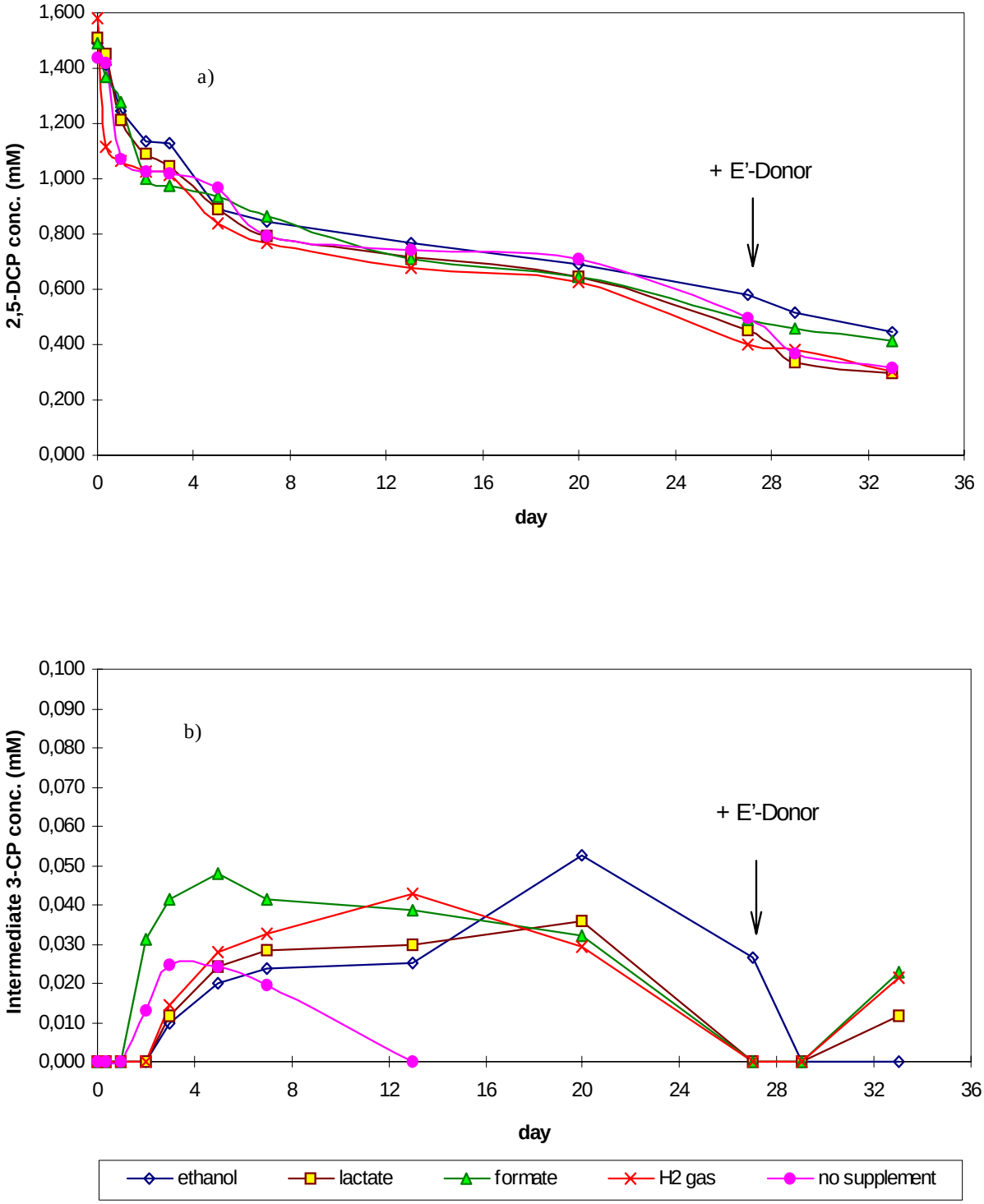


Fig. 3.32 Transformation of 2,5-DCP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 2,5-DCP (b) concentration of 3-CP (intermediate from dechlorination of 2,5-DCP).

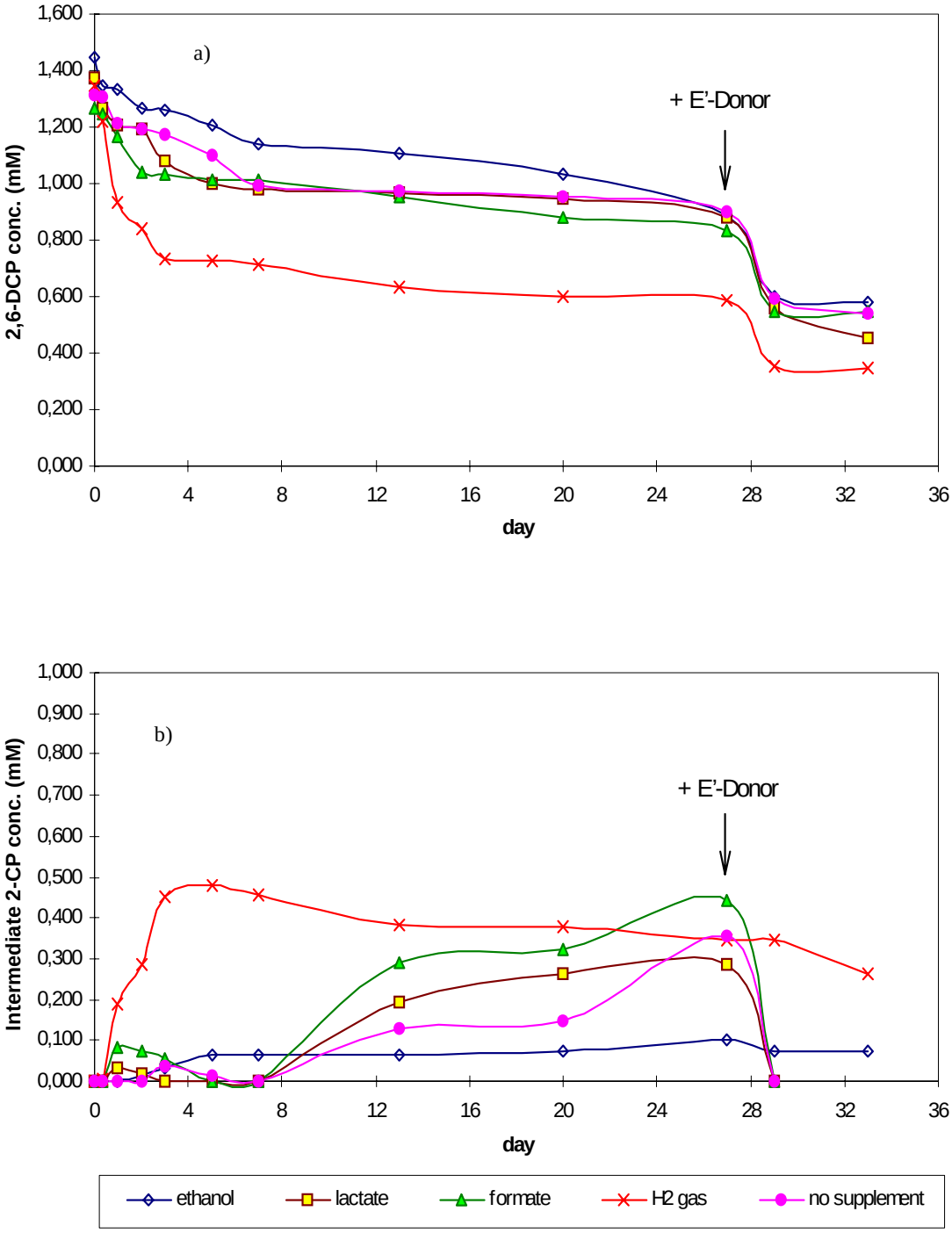


Fig. 3.33 Transformation of 2,6-DCP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 2,6-DCP (b) concentration of 2-CP (intermediate from dechlorination of 2,6-DCP).

Either in the presence or absence of an electron donor, 2,4,5-TCP was sequentially dechlorinated via 3,4-DCP to 3-CP (Fig. 3.34 a-c) and most of it was transformed when incubated with lactate or H_2 gas (Table

3.3). The accumulation of 3,4-DCP in the samples which had ethanol as electron donor and accumulation of 3-CP in samples which were dosed with lactate and formate was observed during the incubation (Fig. 3.34 b, c). Transformation of other TCPs was a little different from that of 2,4,5-TCP. In the samples which were incubated with H_2 gas, 2,3,6-TCP was sequentially dechlorinated to 2,5-DCP and 3-CP (Fig 3.35 a) whereas 2,3,6-TCP in other samples was immediately double dechlorinated to 3-CP, which was further transformed by the cultures (Fig. 3.36). For the removal of 3-CP, non-amended samples and samples incubated with lactate showed the highest transformation efficiency (Table 3.3). The cultures required approx. 27 days to remove 2,4,6-TCP from the samples when H_2 gas was used as an electron donor (Fig 3.35 b). Besides 4-CP, 2,4-DCP was found as one of the intermediates of 2,4,6-TCP transformation by 2-CP degrading cultures in samples which were incubated with ethanol and lactate (Fig. 3.37). 2,4,6-TCP in other samples was rapidly dechlorinated to 4-CP, which was completely removed within 3 days

Most of the PCP was removed at the begining of the incubation like the transformation of PCP in samples amended with glucose (Fig. 3.38 a). Among four electron donors, ethanol showed the greatest efficiency on PCP transformation (Table 3.3). Many intermediates detected by HPLC were found from the transformation of PCP. Based on the retention time, two interesting intermediates (designed intermediate 3 and intermediate 4) were assumed to be one of the less-chlorinated phenolic compounds although none of their retention times matched with those of available reference compounds. Intermediate 4 was suspected to be tetrachlorophenol (TeCP) because its retention time was between TCP and PCP. Intermediate 3, whose retention time was between phenol and 2-CP, might have been catechol, one of the products from hydrolytic dechlorination.

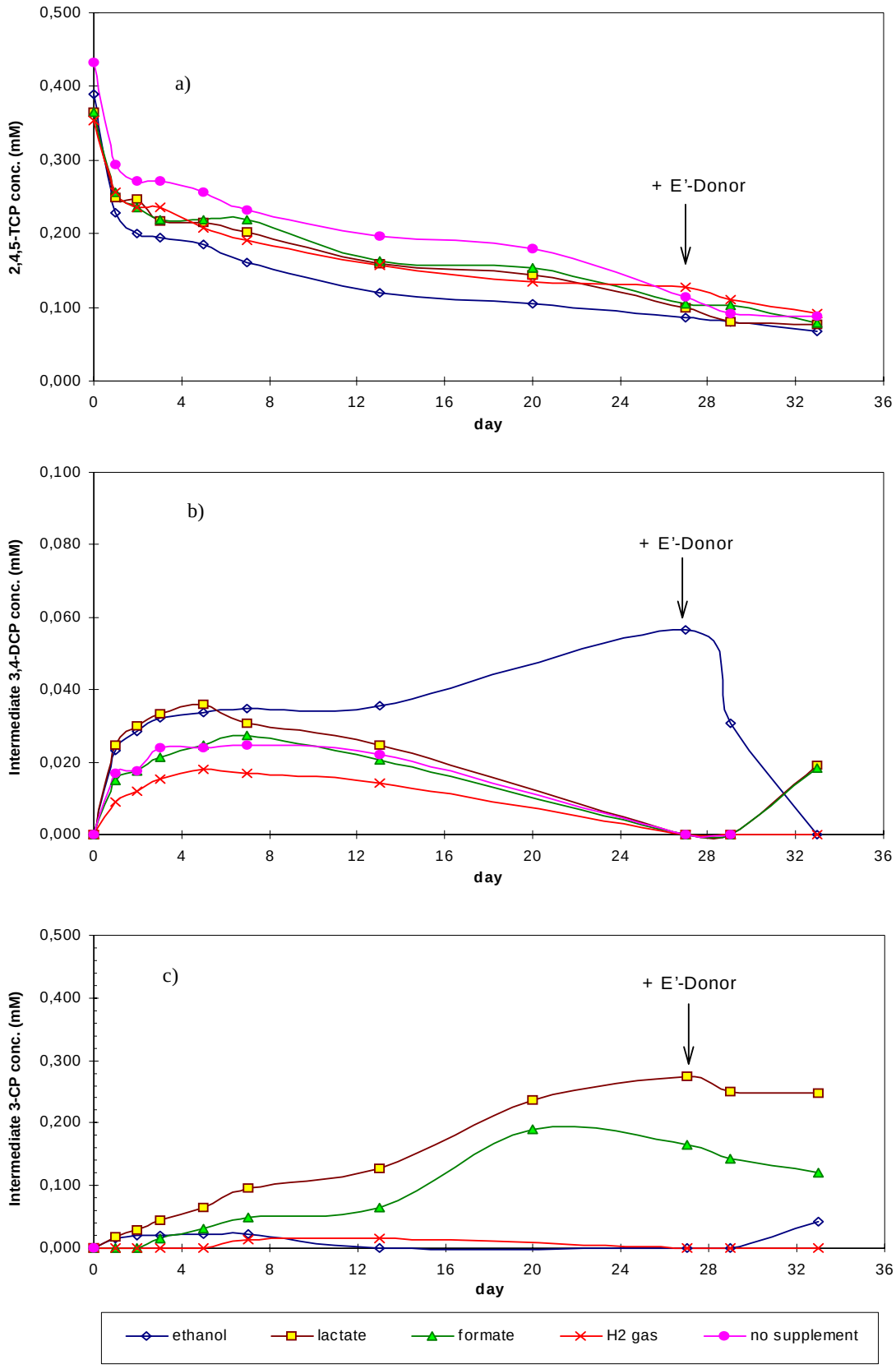
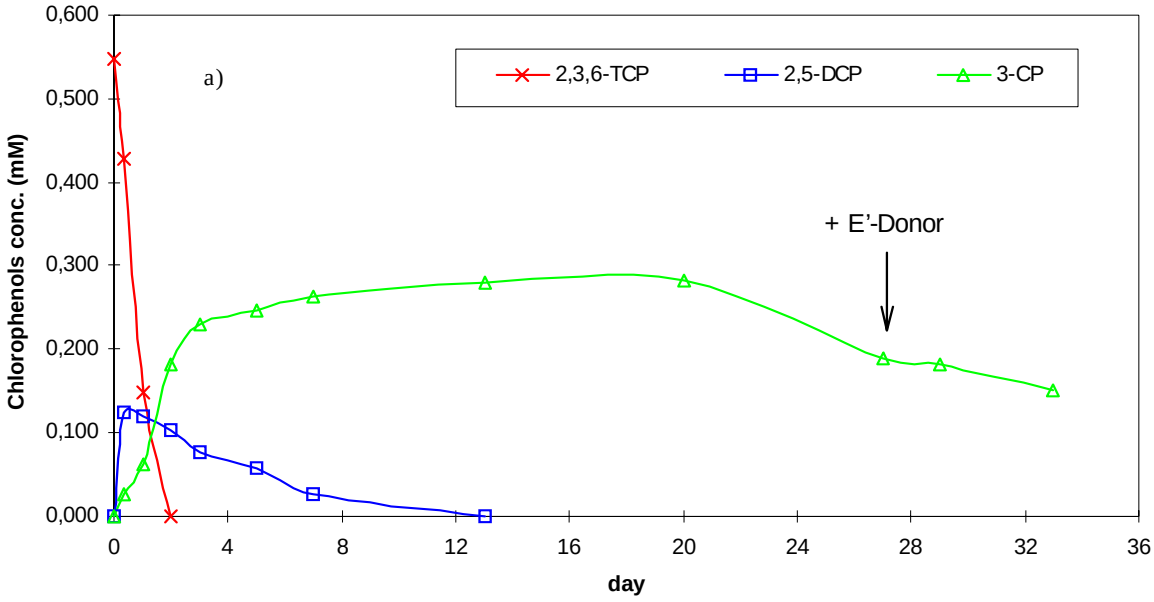


Fig. 3.34 Transformation of 2,4,5-TCP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 2,4,5-TCP (b, c) concentration of 3,4-DCP and 3-CP (intermediates from dechlorination of 2,4,5-TCP).



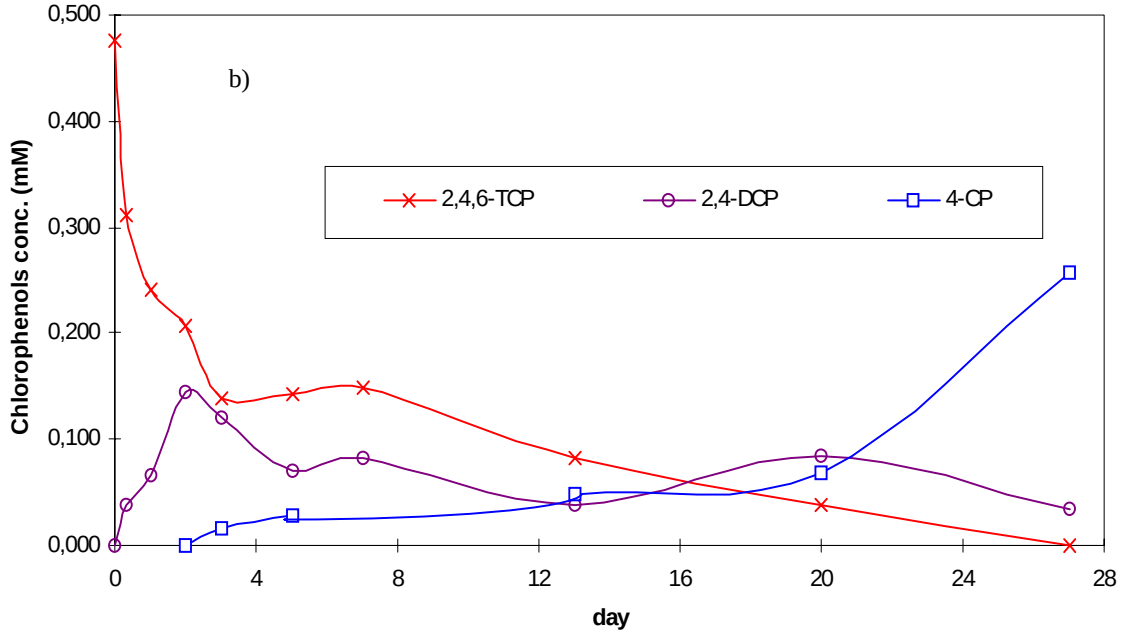


Fig. 3.35 Transformation of 2,3,6-TCP (a) and 2,4,6-TCP (b) by 2-CP degrading cultures amended with H_2 gas as electron donor.

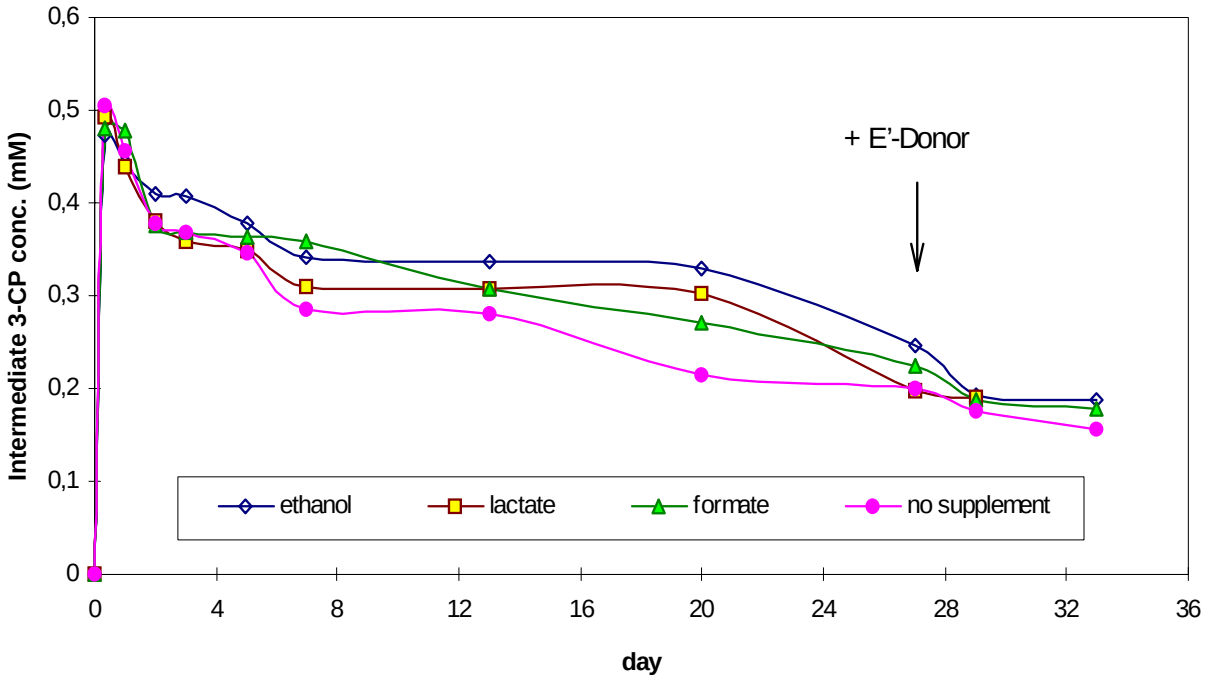


Fig. 3.36 Concentration of 3-CP (intermediate from the dechlorination of 2,3,6-TCP) in 2-CP degrading cultures amended with various electron donors.

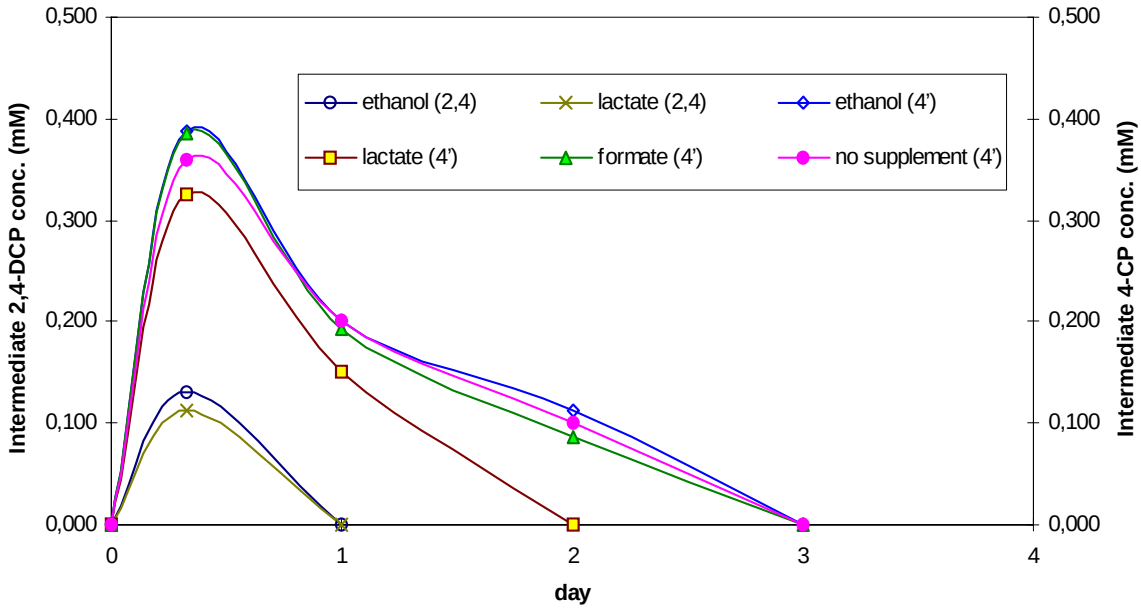


Fig. 3.37 Concentration of 2,4-DCP and 4-CP (intermediates from the dechlorination of 2,4,6-TCP) in 2-CP degrading cultures amended with various electron donors.

The amount of these two intermediates, in terms of peak area, in each sample is shown in Figure 3.38 (b, c). It should be noted that phenol was found as the intermediate in samples incubated with H_2 gas instead of

intermediate 3 while in other samples no phenol was detected. The highest amount of intermediate 4 was found in samples amended with ethanol and the highest amount of intermediate 3 was observed in non-amended samples.

On day 27 of the experiment the same amount of each of the electron donors as initially was added to samples in which the transformation was not complete. In non-amended cultures, 5 mM of glucose was used as electron donor. The decline of each chlorophenol and of their intermediates was obviously seen in almost every sample after a few days of incubation (Fig. 3.29-3.38). These results confirmed the stimulatory effect of electron donors on the dechlorination of chlorophenols.

3.10 Biosorption of PCP by anaerobic granular sludge

In the evaluation of the potential of 2-CP degrading granules for degradation of PCP in the presence of various electron donors (see 3.9.1 and 3.9.2), it was observed that the amount of intermediates evtl. chloride ions did not correspond with the quantity of PCP that disappeared. According to a number of studies PCP adsorbed to anaerobic granular sludge (Kennedy et al., 1992; Kennedy and Pham, 1995). It was assumed that some PCP was adsorbed by the 2-CP degrading granules. To confirm this hypothesis, the biosorption of PCP to 2-CP degrading granules at different concentrations was investigated using batch serum bottle tests.

Figure 3.39 illustrates the concentration of PCP that was found in supernatant of control and tested samples at each time interval (initial PCP conc. was 40 mg/l). Approximately 30 % of PCP that was added into the samples was immediately adsorbed to the biomass and/or glass bottles. The difference between PCP concentration in supernatant of the

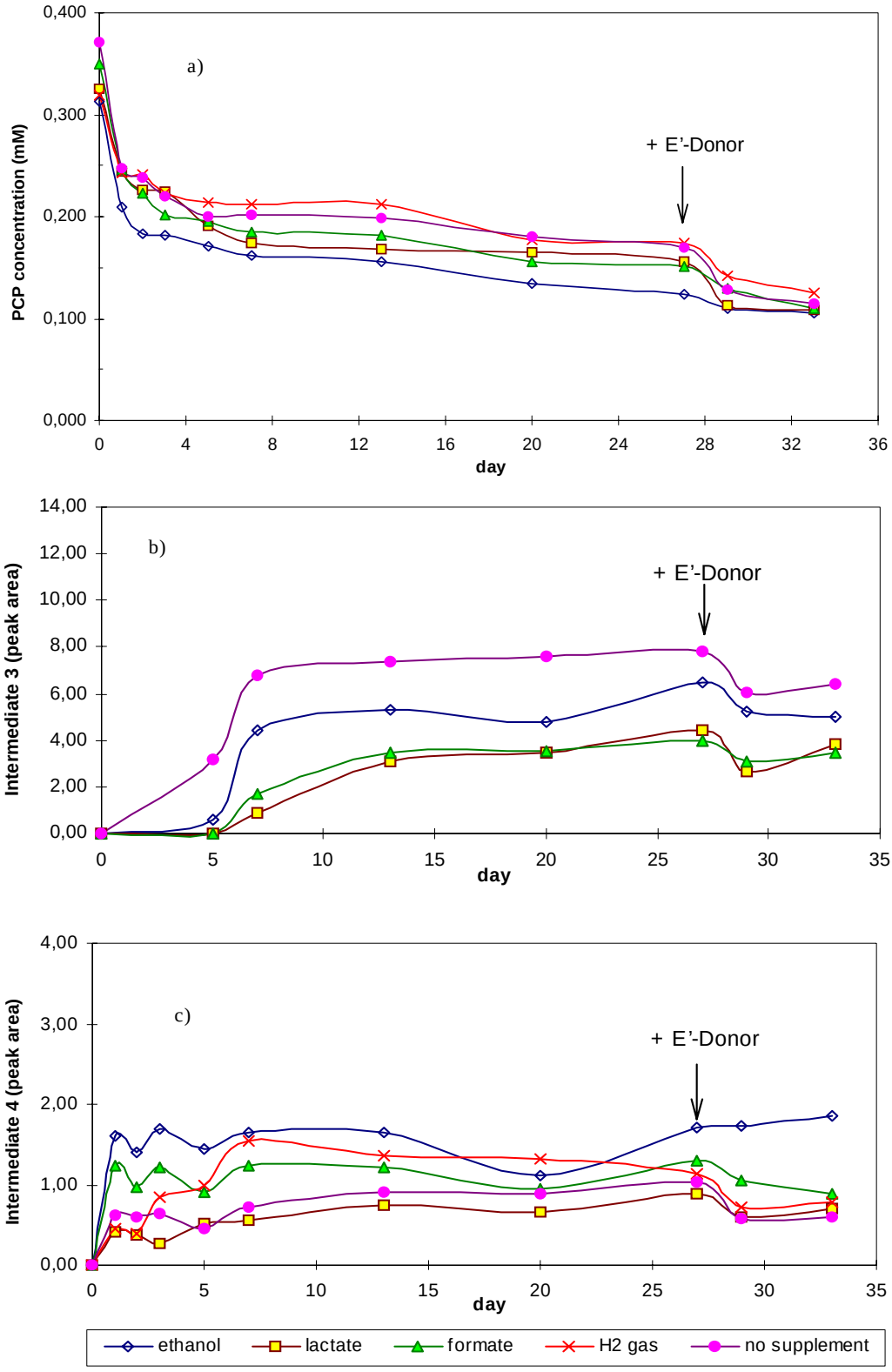


Fig. 3.38 Transformation of PCP by 2-CP degrading cultures amended with various electron donors. (a) Concentration of PCP; (b, c). Amount of intermediate 3 and intermediate 4, expressed in form of peak area, occured during the transformation of PCP by 2-CP degrading cultures (Intermediate 3 might be catechol, intermediate 4 might be tetrachlorophenol).

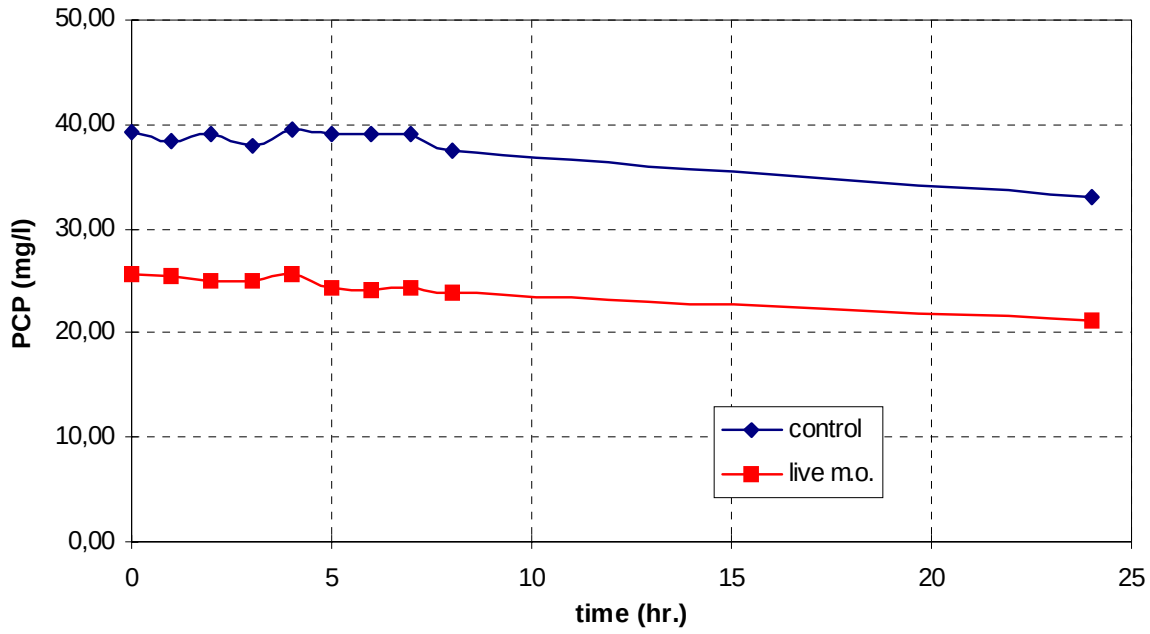


Fig. 3.39 Concentration of PCP in supernatant of control and tested biomass at each time interval.

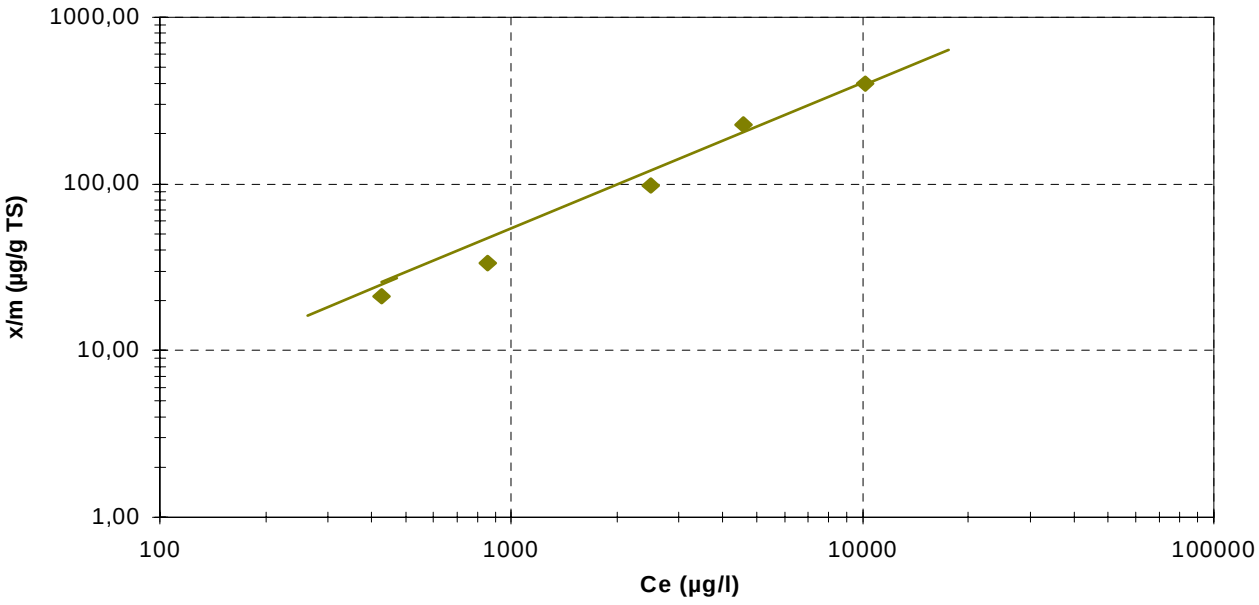


Fig. 3.40 Biosorption of PCP by living anaerobic granular biomass at 35°C.

control and the tested samples was relatively constant during 24 hours of incubation. The concentration of PCP used for determination of the biosorption isotherm of PCP was between 0.5-20 mg/l. Equilibrium conditions were determined after 3 hours of contact time to avoid the transformation of PCP by the tested granules although the results from the above test showed that the concentration of PCP in tested samples was constant over 24 hours of contact time. Figure 3.40 shows live anaerobic granular biomass biosorption data by the Freudlich equation isotherm. Non-linear least squares analysis were performed on the equilibrium data to determine the Freundlich constant, which gave *K* (Freundlich constant) = 5.48 x 10^{-2} and 1/*n* (exponent) = 0.97. The isotherm fitted the Freundlich equation very well, with a correlation coefficient greater than 0.9.

3 RESULTS

3.1 Performance of an UASB reactor for the treatment of glucose containing synthetic wastewater (reactor R 1)

3.1.1 COD and TKN removal efficiency

A UASB reactor (R 1), inoculated with granular sludge from a full-scale UASB reactor treating brewery wastewater, was fed with an artificial glucose-containing wastewater to give a HRT of 8 h. The HRT was changed to 24 h after the pellets were partially inactivated by high temperature (failure of the thermostat). When the reactor removed over 80% of the total COD from the wastewater, the organic loading rate was increased stepwise. Figure 3.1 depicts the relationship between removal efficiency (COD and TKN) and organic loading rate.

At a constant HRT of 8 h, the glucose concentration in wastewater was varied from 1 to 5 g/l, corresponding to a organic loading rate of 5-15 g COD/l, d. A COD removal efficiency ≥ 90 % was achieved when the reactor was operated at organic loading rates lower than 9 g COD/l, d. The COD removal efficiency decreased to 77 % when the organic loading rate was increased to 12 g COD/l, d.

During the experiment, the temperature of the reactor was accidentially heated 35 °C to 80°C for about 4-5 hours, due to the breakdown of the thermostat. Flotation of granules (due to increased CO_2 evolution by a temperature shift of the equilibrium $CO_3^{=}$ $HCO_3^{=}$ CO_2) was observed immediately after the occurence of

shock heating. In an attempt to restore the biological activity in the system and to reduce the loss of granules, the upflow velocity in the reactor was reduced from 8.4 to approx. 5 m/h and medium addition was omitted until the quantity of floating granules was reduced significantly. The recovery of granules began on the fifth day after shock heating. The reactor was then fed again and the organic loading rate was slowly increased.

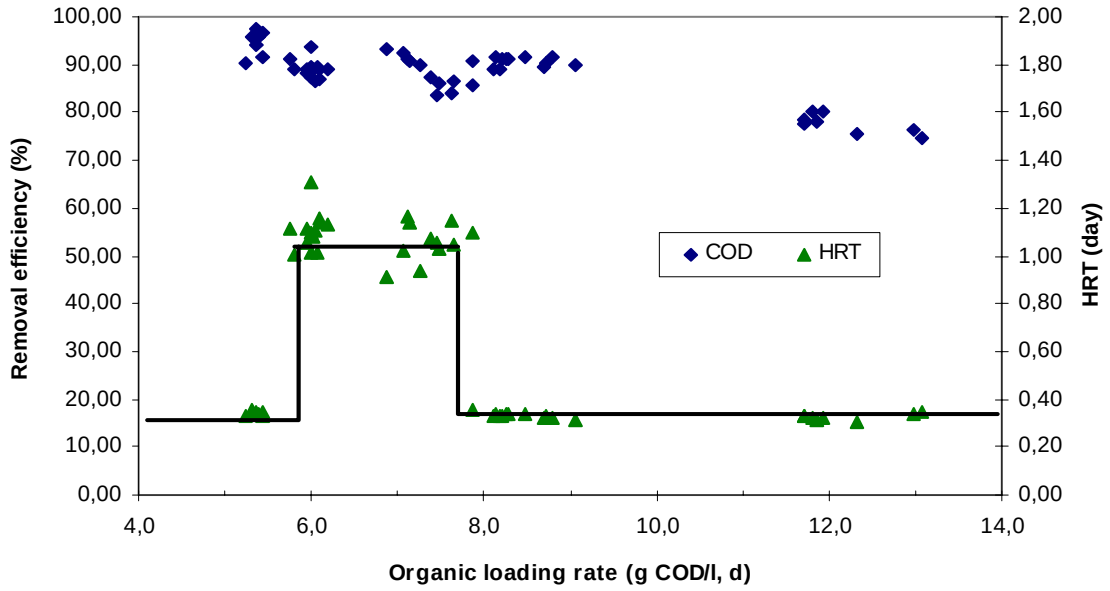


Fig. 3.1 HRT and total COD removal efficiency of reactor R 1 at different organic loading rates.

After the heat shock the effectiveness of granules for anaerobic digestion was greatly diminished. A COD removal efficiency of 90 % was hardly attained even at a loading rate as low as 5 g COD/l, d. The granules began to clump and stick together when the glucose concentration in the medium was higher than 2 g/l. Consequently, the upflow velocity of the reactor was raised to its preceding value of 8.4 m/h to enhance the mixing in reactor and the HRT was increased to 15 h in order to reduce the loading and to improve the removal efficiency. However, a satisfying removal efficiency (over 80 %) was not achieved until the HRT of the reactor was increased to 24 h. The system could remove approx. 90 % of the COD in wastewater at the provided loading rate between 6-8 g COD/l, d. When the organic loading was higher than 9 g COD/l, d the removal efficiency dropped drastically to around 50 % and only half of the expected methane was produced (data not shown). Many fatty acids e.g. acetate, propionate, butyrate were found in the effluent indicating that the reduction of removal efficiency was the result of an imbalance between acid formers and acetogenic and methane bacteria in the system.

No nitrogen was removed during anaerobic degradation, except for that portion used for growth of the bacteria.

3.1.2 Methane production

The change of the methane production rate during the experiment is shown in Figure 3.2. Anaerobic digestion of sugars like glucose can theoretically yield about 0.35 l_{STP} of methane per 1 g of COD, as derived from the Buswell equation (Eq. 1) or the conversion stoichiometry.

$$C_nH_aO_b + (n - \frac{a}{4} - \frac{b}{2})\, H_2O \longrightarrow (\frac{n}{2} - \frac{a}{8} + \frac{b}{4})\, CO_2 + (\frac{n}{2} + \frac{a}{8} - \frac{b}{4})\, CH_4 \quad \text{..... (1)}$$

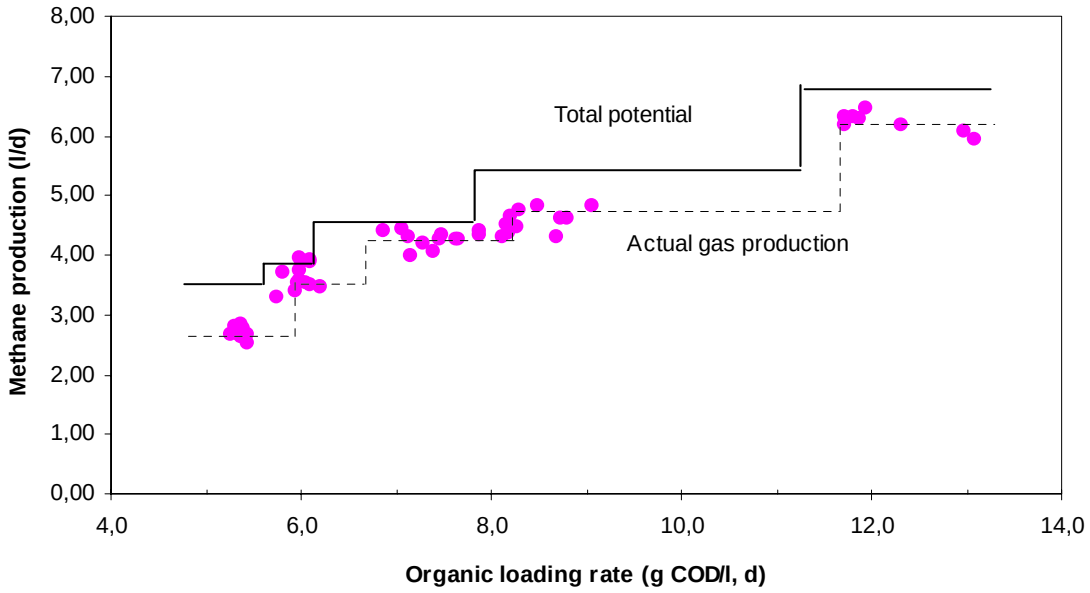


Fig. 3.2 Actual and potential methane production from reactor R 1 at different organic loading rates.

The total potential of the methane production rates, calculated theoretically from the COD load was drawn as a solid line in Figure 3.2. The actual methane production at each organic loading rate was 10-27 % less than the theoretical value. The difference between actual methane production and theoretical value was only 7-10 % when the HRT of the reactor was increased to 24 h. Usually, gas from anaerobic digestion of mixed substrates is composed of 70-85 % methane, 15-30 % carbon dioxide and a small amount of hydrogen sulfide, H_2 and other

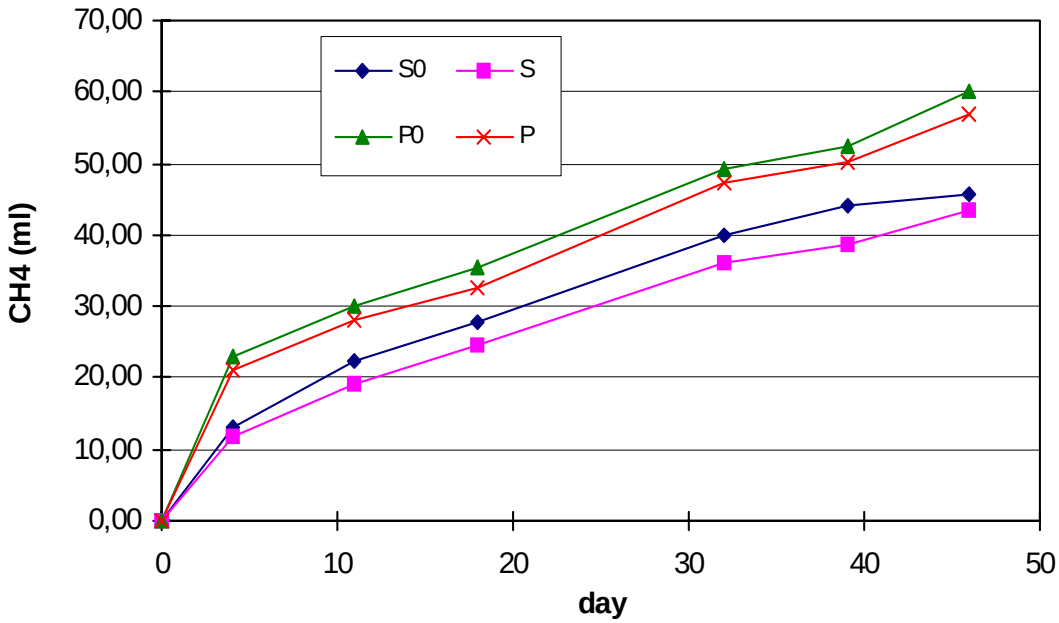
gases. Methane measurement of the effluent gases from reactor R 1 revealed that the biogas contained about 65 % methane, as typically obtained, when carbohydrates were fermented.

3.2 Acclimation and enrichment of chlorophenol-degrading granules

3.2.1 Preliminary test

The ability of unacclimated sludge to degrade monochlorophenol was examined in 120 ml serum bottles by incubating glucose-degrading sludge pellets from reactor R 1 with 1 mM of 2-chlorophenol (2-CP) as initial concentration in 20 ml phosphate buffer, pH 7.5, at 35°C. The control samples that did not contain 2-CP were incubated under the same condition. The 2-CP was selected as a representative of monochlorophenols because it was reported to be degraded by undefined cultures in several systems (e.g., Boyd & Shelton, 1984; Gibson & Suflita, 1986; Dietrich & Winter, 1990; Mohn & Kennedy, 1992; Flora et al., 1994) and even by a pure culture, strain 2CP-1 (Cole et al., 1994).

Two forms of unacclimated inoculum, granular sludge from R 1 and suspended sludge from a sewage digester, were used in this test and methane production was monitored for over 40 days. Within 46 days the



methane amount in granular sludge (P) and suspended sludge (S)

Fig. 3.3 Methane production of suspended (S) and granular sludge (P), incubated at 35°C with 1 mM of 2-CP in 20 ml phosphate buffer, pH 7.5. The methane production of the control samples

that did not contain 2-CP (S_0, P_0) was also measured.

samples has reached 54 and 43 ml, respectively (Fig. 3.3). The result showed that both sludge sources retained their potential to form methane in the presence of toxic substances like 1 mM 2-CP. Apparently no methane was formed from 2-CP.

3.2.2 Anaerobic biodegradation potential of unacclimated sludge for different substituted monochlorophenols (2-CP, 3-CP, 4-CP)

From methane measurement of the previous experiment, it could not be shown whether 2-CP was transformed or degraded by these sludges. Therefore, the quantitative determination of chlorophenol or phenol concentrations by high performance liquid chromatography (HPLC) was established in order to verify a biodegradation of chlorophenols or a biotransformation by reductive dehalogenation.

To examine the anaerobic biodegradation potential of unacclimated sludge for different monochlorophenols (2-CP, 3-CP, and 4-CP) or the inhibitory effect of these substances, glucose-degrading granules were incubated with various concentrations of each MCP ranging from 0.5 mM to 10 mM. A control without toxicant was included as a basis for comparison. Total methane production and MCP disappearance were monitored for 9 weeks.

Cumulative methane production from each sample is shown in Figure 3.4 (a-c). For each MCP, gas production gradually decreased as the concentration of the three MCPs increased. Only little methane was produced in all assays (not more than 40 ml/g TS) when the MCP concentration was over 2 mM. While for each MCP a similar methane production profile was obtained, the biotransformation of the MCPs differed significantly. The transformation of 3-CP occurred mostly in the first 2-3 weeks of incubation. After that the concentration of 3-CP in each sample was more or less unchanged whereas 2-CP and 4-CP were further transformed until the 5^{th} week. Table 3.1 shows % transformation of each MCP by the glucose-degrading granules after day 35 of incubation.

Table 3.1 Percentage of each MCP transformed by glucose-degrading granules until day 35 of incubation.

MCP concentration	**transformation (%)**		
	2-CP	***3-CP***	***4-CP***
0.5 mM	100	43.70	25.60
1.0 mM	100	38	21.40
2.0 mM	34.30	30.30	15.60
5.0 mM	22.54	14.86	7.28
7.5 mM	14.95	11.76	7.28
10.0 mM	10.32	5.84	5.50

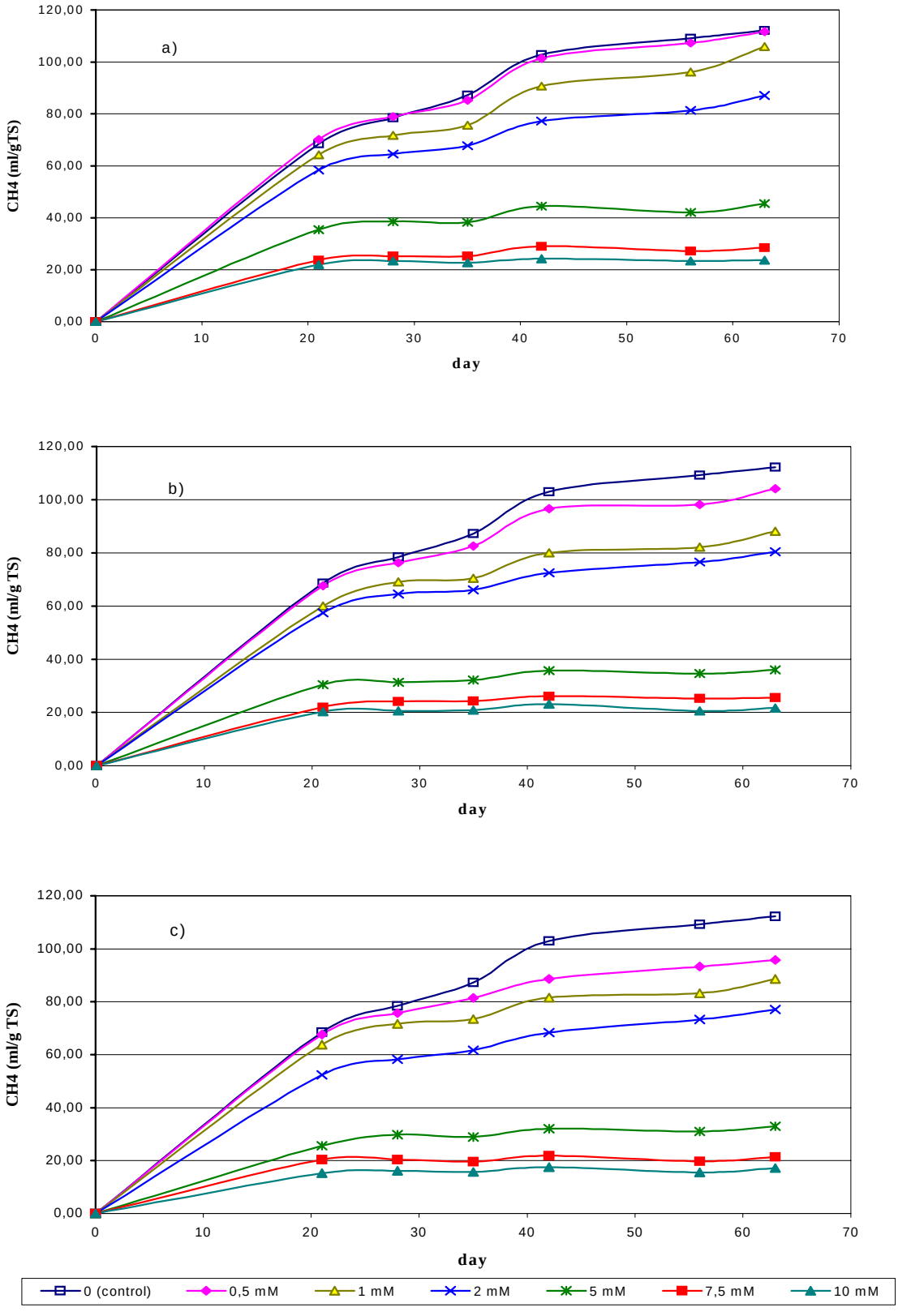
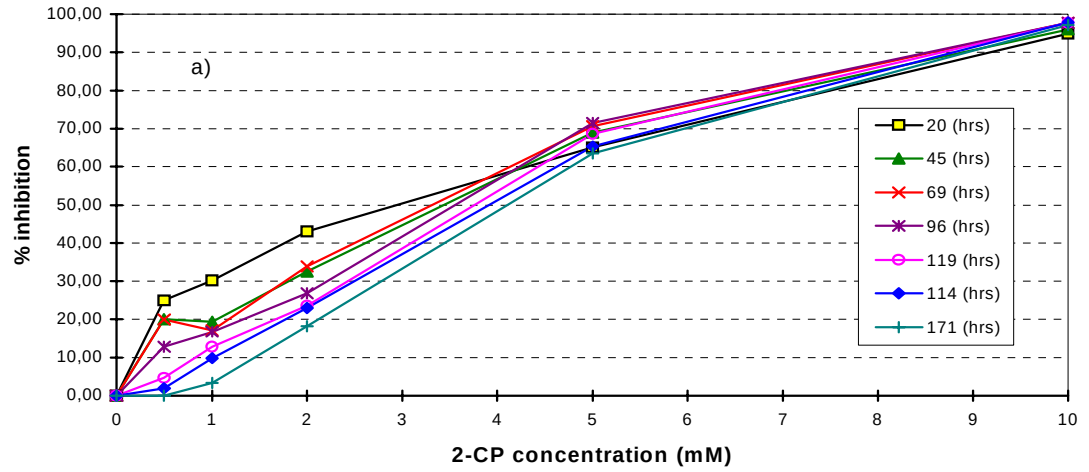


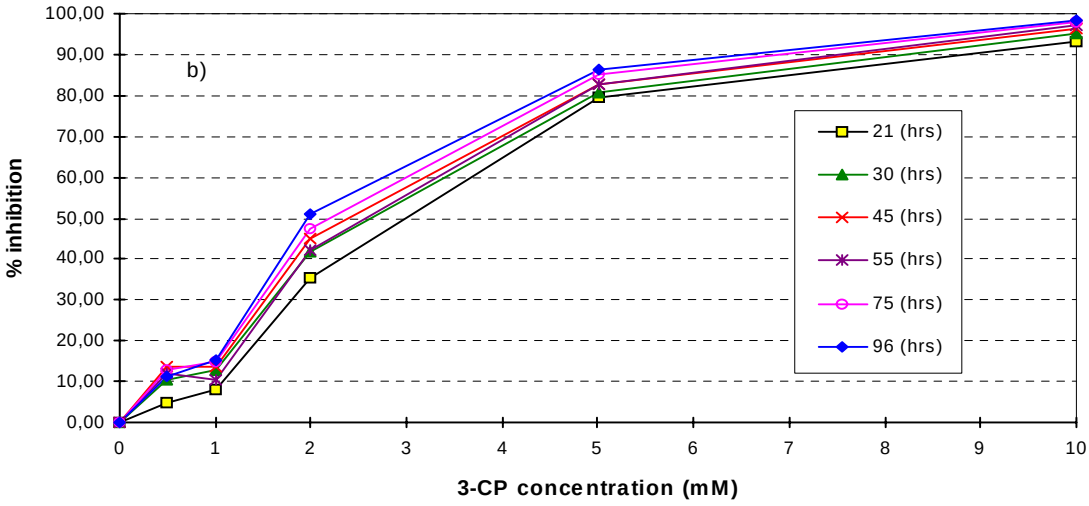
Fig. 3.4 Total methane production of granular sludge from an UASB reactor fed with glucose containing synthetic wastewater, which was supplemented with various concentrations of 2-CP (a), 3-CP (b) and 4-CP (c) in 120 ml serum bottles.

From the results of Table 3.1, it is apparent that the position of the chlorine substituent affects the degradation or dehalogenation of MCP. 2-CP was transformed to a higher degree than 3-CP and 4-CP. Especially at the concentration of 0.5 and 1 mM, 2-CP and phenol, the transformation product from reductive dechlorination, was completely degraded within 35 days whereas 3-CP and 4-CP were transformed only to an extend of 38-44 % and 21-26 %, respectively.

3.2.3 Toxicity of monochlorophenols on unacclimated sludge

Anaerobic toxicity assays (ATA) were performed to evaluate the toxicity of various concentrations of monochlorophenols. The initial concentations of MCP were 0.5, 1, 2, 5, and 10 mM. As can be seen from Figure 3.5 a-c, toxicity increased with increasing dose of toxicant until gas production ceased (100 % inhibition). At the MCP concentrations of ≥ 5 mM, methane production was more than 60 % inhibited. The close agreement between inhibition curves of each concentration of 3-CP (Fig. 3.5 b) and 4-CP (Fig. 3.5 c) at different test times indicated that there was no recovery from the toxic impacts during the incubation period. On the other hand, the inhibition curves of 2-CP at concentrations up to 2 mM (Fig. 3.5 a) showed a slight recovery from the toxic effect of 2-CP which improved with time. For example at a concentration of 2 mM, methane production was about 40 % inhibited in the first 20 hours of the incubation but after 5 days (120 hrs.) the inhibition was only 23 %. The inhibitory effect of the chlorophenols was about 10 % decreased when the old samples were supplemented with acetate-propionate solution and MCP again. From this result, it can be assumed that degradation of MCP had occured or that the organisms did acclimate or adapt to the presence of that chlorophenol isomer. This phenomenon could obviously be seen in the sample which was incubated with 5 mM 2-CP after new acetate-propionate solution and 2-CP were added





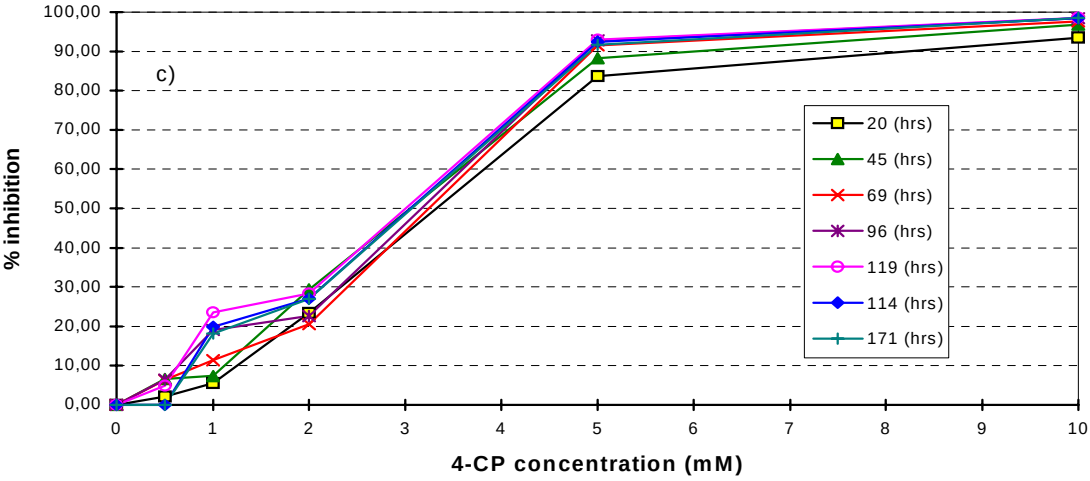


Fig. 3.5 Inhibition of methane production from an acetate-propionate solution with increasing incubation time, when unacclimated granular sludge was incubated with 5 different concentrations of (a) 2-CP, (b) 3-CP and (c) 4-CP at 37°C.

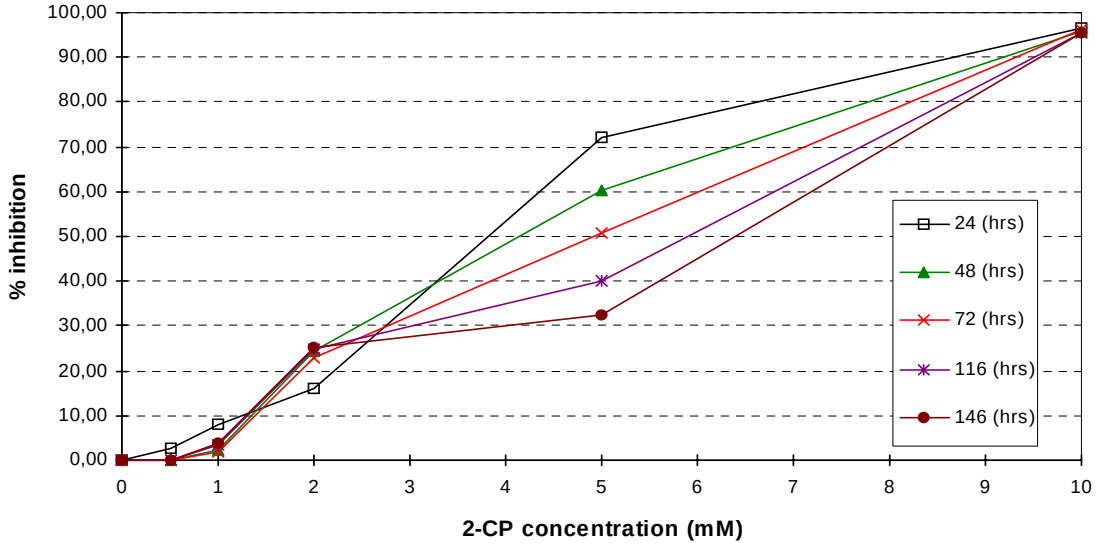


Fig. 3.6 Inhibition of methane production from an acetate-propionate solution by 2-CP in unacclimated granular sludge cultures after substrate and 2-CP were added a second time.

(Fig. 3.6). The inhibition of methane production was 50 % less than in the preceding incubation. In Figure 3.7 the inhibition after 48 h of incubation for different concentrations of each MCP of methane production was compared. It seemed likely that 3-CP had the most toxic effect on methane production among the three isomers of MCP.

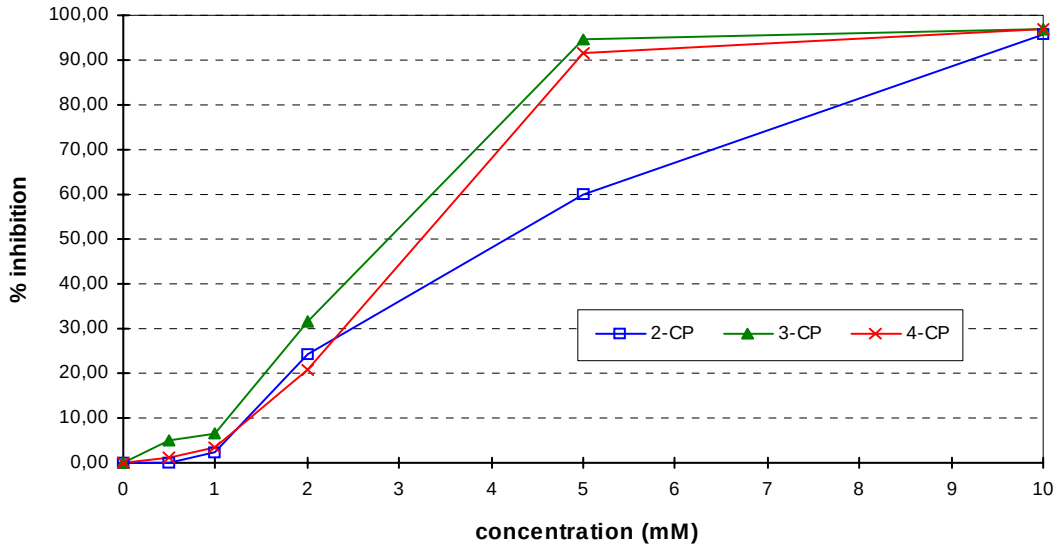


Fig. 3.7 Inhibition of methane production from acetate-propionate solution by each monochlorophenol in unacclimated granular sludge cultures after incubation for 48 hours.

3.2.4 Effect of organic substrates on dechlorination of monochlorophenol

Results from preceding experiments demonstrated that 2-CP, 3-CP, and 4-CP at concentration up to 1 mM could either be degraded by unacclimated sludge or their effect on methane production from other substrates was relatively low (less than 10 % inhibition of gas production). In addition, there was almost no difference in degradation and the inhibitory pattern of methane production for each MCP between 0.5 mM and 1 mM. Thus, 1 mM of MCP, a safe level based on the results of the toxicity assays, was selected to use as an initial concentration for selection of MCP-degrading granules and as a basic concentration for further experiments.

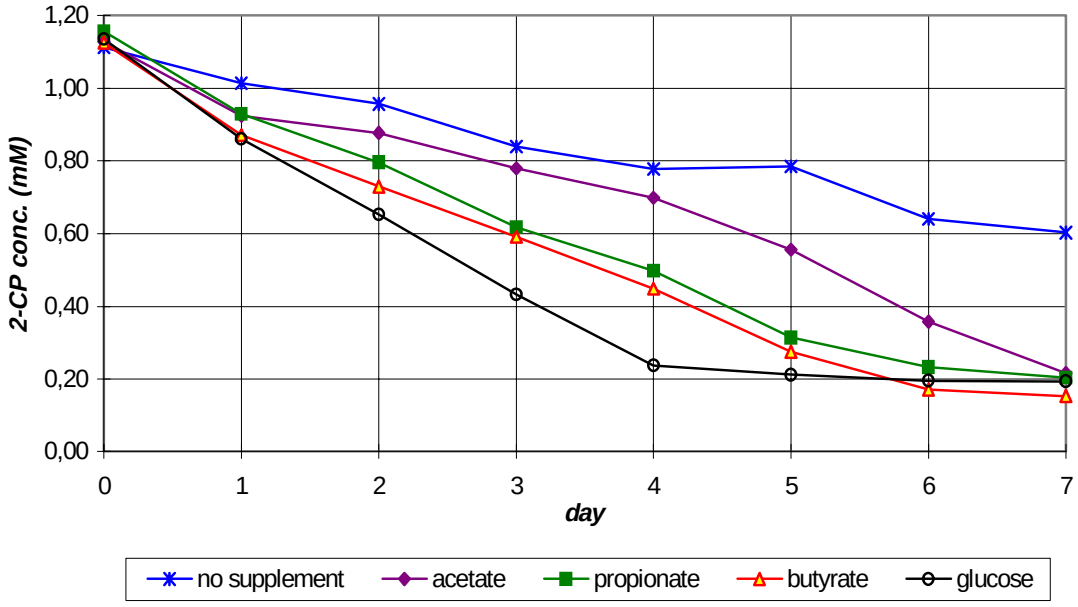


Fig. 3.8 Effect of organic substrates on the dechlorination of 2-chlorophenol.

The stimulatory effect of organic substrates on anaerobic dehalogenation of haloaromatic substances had been reported in many studies (Kuhn et al., 1990; Nies & Vogel, 1990; Gibson & Suflita, 1990; Hendriksen et al., 1992). For this reason, it would be useful to study whether supplemental organic substrates also have positive influence on acclimatization to dechlorination of MCP in granular sludge or not. Glucose-degrading granules were dosed with 1 mM 2-CP, 3-CP or 4-CP and incubated anaerobically together with 2 mM of acetate, propionate, butyrate, or glucose. A control sample (no additional carbon supplement) was similarly incubated. Methane production and depletion of both, 2-CP and the organic substrates, were analysed. After 7 days of incubation, 2-CP in all samples was almost completely dechlorinated (Fig. 3.8). All of the 4 different organic substrates enhanced the dechlorination rate of 2-CP. Glucose had the greatest stimulatory effect. The dechlorination in the samples which were dosed with 3-CP or 4-CP occured only to an extend of less than 10 %, independently of whether any of the organic substrates was amended or not. For further experiments glucose was

used as a carbon source to stimulate the dechlorination of 2-CP, 3-CP and 4-CP during acclimatization of glucose-degrading granules to each MCP. Unfortunately, the transformation of 3-CP and 4-CP in the enrichment cultures did not improve after 3 months of acclimation time. Hence, only an enrichment culture which was acclimated to 2-CP was available for further experimentations.

3.3 Optimal conditions for operating a continuous UASB reactor for treatment of a 2-CP containing synthetic wastewater

3.3.1 Optimum concentration of glucose amendment

Five concentrations of glucose (2 mM, 5 mM, 10 mM, 15 mM, and 20 mM) were supplemented to find out the most suitable concentration for dechlorination of 1 mM of 2-CP. As shown in Figure 3.9, the dechlorination rates of 2-CP for all concentrations of glucose were not significantly different, especially in the first 8 hours of incubation. The control sample which obtained no glucose was incubated in parallel to confirm the stimulatory effect of glucose on dechlorination.

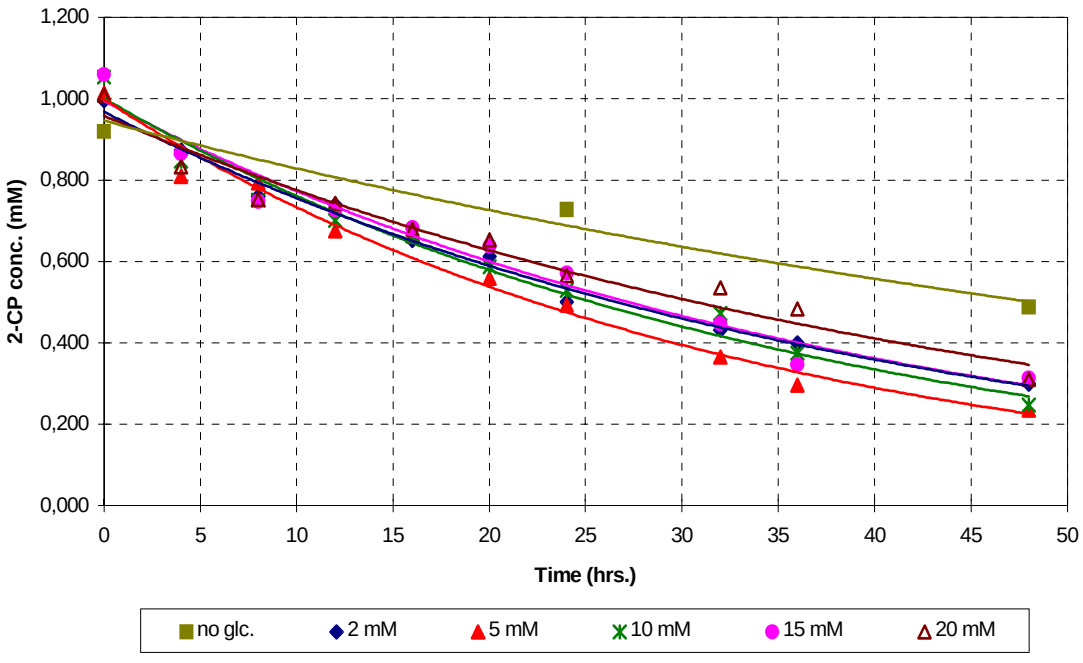


Fig. 3.9 Dechlorination of 2-CP in the presence of various glucose concentrations.

By using the non-linear regression method, the transformation rates of 2-CP could be compared. The initial velocity of 2-CP disappearance increased in the sequence from 5 mM > 10 mM > 15 mM ≥ 2 mM

> 20 mM, and rates calculated for 4 hours of incubation were 0.48, 0.43, 0.41, 0.38, and 0,32 µM/min, respectively. Considering the rates of dechlorination, 5 mM of glucose was the optimal concentration for dechlorination of 1 mM of 2-CP. The good adaptation of this enrichment culture to 2-CP can also be seen from Figure 3.9, since 2-CP at the same concentration of glucose (control and 2 mM) was more than 50 % dechlorinated within 48 hours, opposed to only 15 - 40 % in the experiment shown in Figure 3.8.

3.3.2 Optimal pH for dechlorination

During determination of the optimal glucose concentration for 2-CP degradation, it was found that dechlorination was strongly dependent on the pH of the medium. One mM of 2-CP was incubated with various concentrations of glucose ranging from 5 mM to 20 mM in two different buffer systems, i.e. bicarbonate (initial pH = 8.5) and phosphate buffer (initial pH = 7.8). The effect of pH on the dehalogenation of 2-CP is shown in Figure 3.10 (a-c).

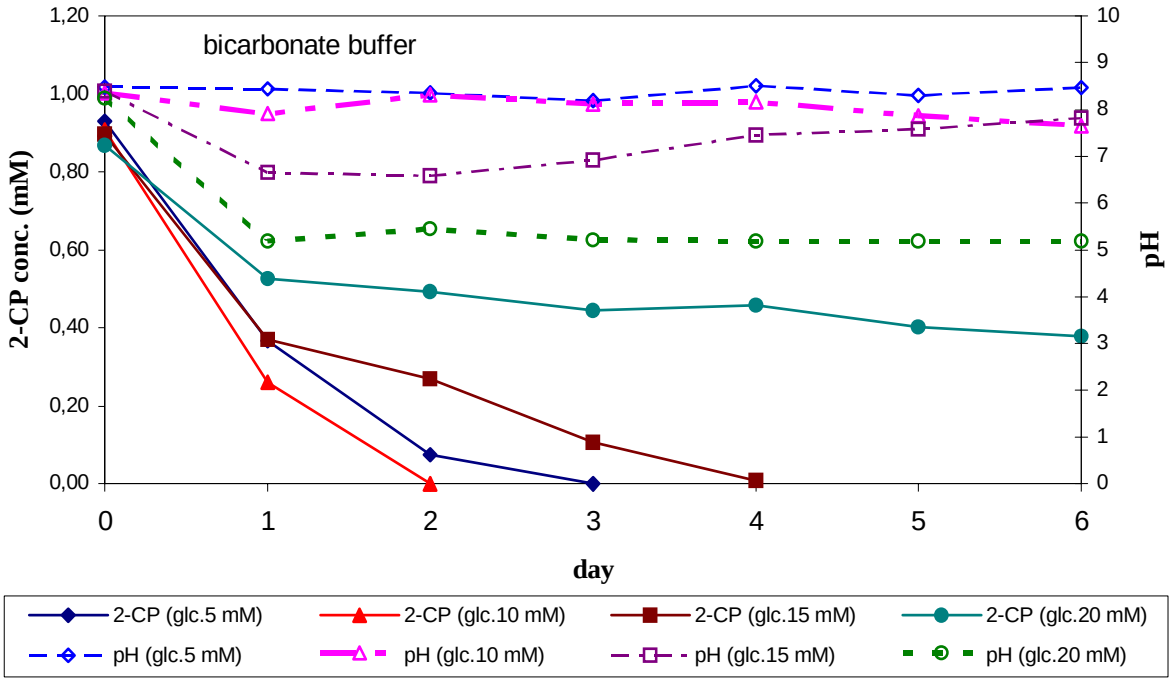


Fig. 3.10 a. Effect of pH on the dechlorination of 1 mM 2-CP in bicarbonate buffer when buffer capacity was not sufficient.

2-CP was completely dechlorinated within 2-4 days when the pH was between 7.0-8.5 (Fig. 3.10 a, b). Dechlorination of 2-CP could also occur when the pH was between 6.5-7.0 but the cultures required more time (5-8 days) for a complete dechlorination of 2-CP (Fig. 3.10 c). It should be noted that 2-CP in samples with a

pH-value below 5.0 could no more be dechlorinated, eventhough the pH of the system was raised to 8.0 (Fig. 3.10 c, arrow). It was assumed that the organisms which have the capability for dechlorination were damaged during 5 days of incubation in low pH condition. The decrease of the pH in the system due to the degradation of glucose indicated that the buffer capacity of the system was not sufficient and then affected the dechlorination of 2-CP. Once the pH of the system was kept stable (i.e. sufficient buffering capacity, Fig. 3.10 b.), there was no marked difference on the dechlorination rates of 2-CP at each concentration of glucose.

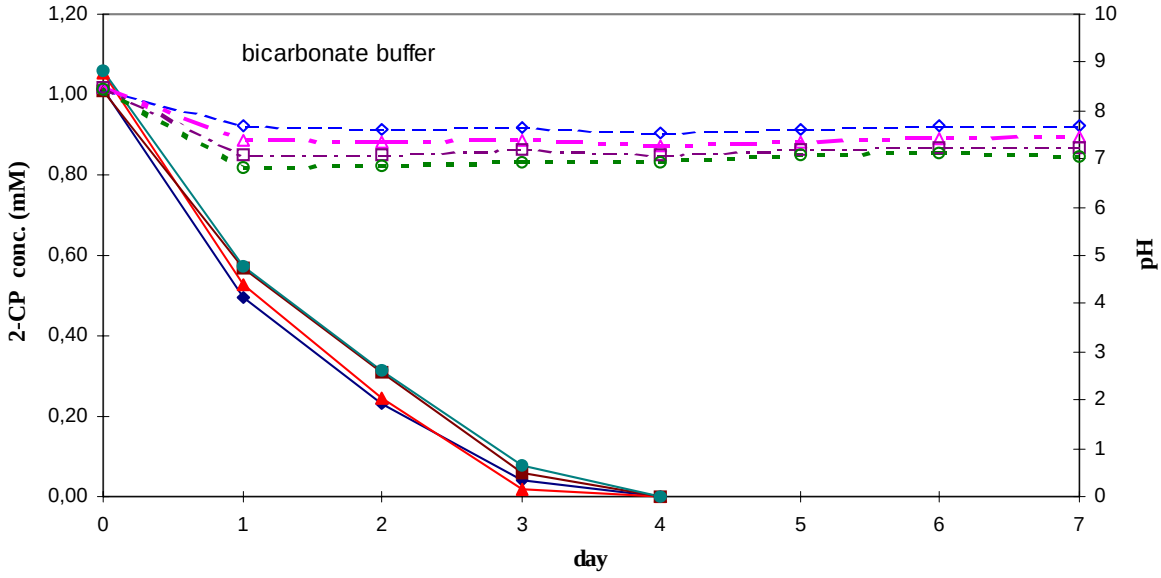


Fig. 3.10 b. Effect of pH on the dechlorination of 1 mM 2-CP in bicarbonate buffer when the buffer capacity was sufficient. All symbols are the same as in Fig. 3.10 a.

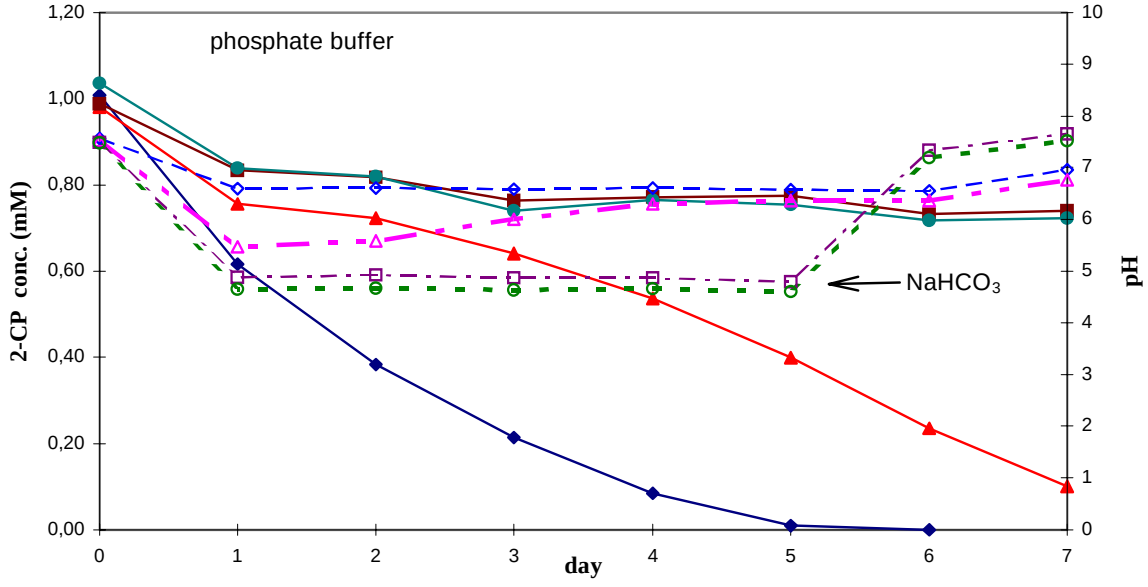


Fig. 3.10 c. Effect of pH on the dechlorination of 1 mM 2-CP in phosphate buffer when the buffer capacity was not sufficient. All symbols are the same as in Fig. 3.10 a

3.4 Performance of the UASB reactor for treatment of 2-CP containing synthetic wastewater (reactor R 2)

The reactor was initially fed 900 mg/l (5 mM) of glucose and 128 mg/l (1 mM) of 2-CP and was operated at a HRT of 5 days to ensure the transformation of 2-CP in the reactor. The flowrate of the recycle stream was maintained at 20 l/h throughout the experiment, resulting in an upflow velocity of approx. 3.1 m/h. Figure 3.11 illustrates the performance of reactor R 2. In Fig. 3.11 a. phenol, 2-CP concentration and HRT, in Fig. 3.11 b. 2-CP loading and % removal, in Fig. 3.11 c. the corresponding COD loading rate and % removal and in Fig. 3.11 d. the total gas production and % methane in biogas are shown.

3*.4.1 Response to hydraulic loading shock*

About 20 days after start up, the 2-CP removal efficiency had reached over 97 % and phenol, an intermediate from dechlorination, was not detectable in the effluent even though the 2-CP concentration in the influent was increased to 1.5 mM (Fig. 3.11 a, b). On day 90 with a stable 2-CP loading rate of 50 mg/l, d the HRT of the system was lowered from 4.5 day to 2.8 day to see whether the high removal efficiency of the startup period could be maintained. The 2-CP removal efficiency dropped to 75 % and seemed difficult to recover to the pre-shock level while the COD removal efficiency was only decreased by 12% (from 90 to 78 %.). Thus, on day 120 the HRT was raised to 4.6 days again, at which 2-CP removal efficiency recovered gradually. Nevertheless, the full recovery of chlorophenol removal activity was not achieved even after 50 days at low 2-CP loading rates as in the startup period.

3.4.2 Response to temperature shock

On day 179, the reactor experienced a temperature shock for about 4-5 hours (from 35°C to 80° C, due to a failure of the thermostat). This temperature change adversely affected

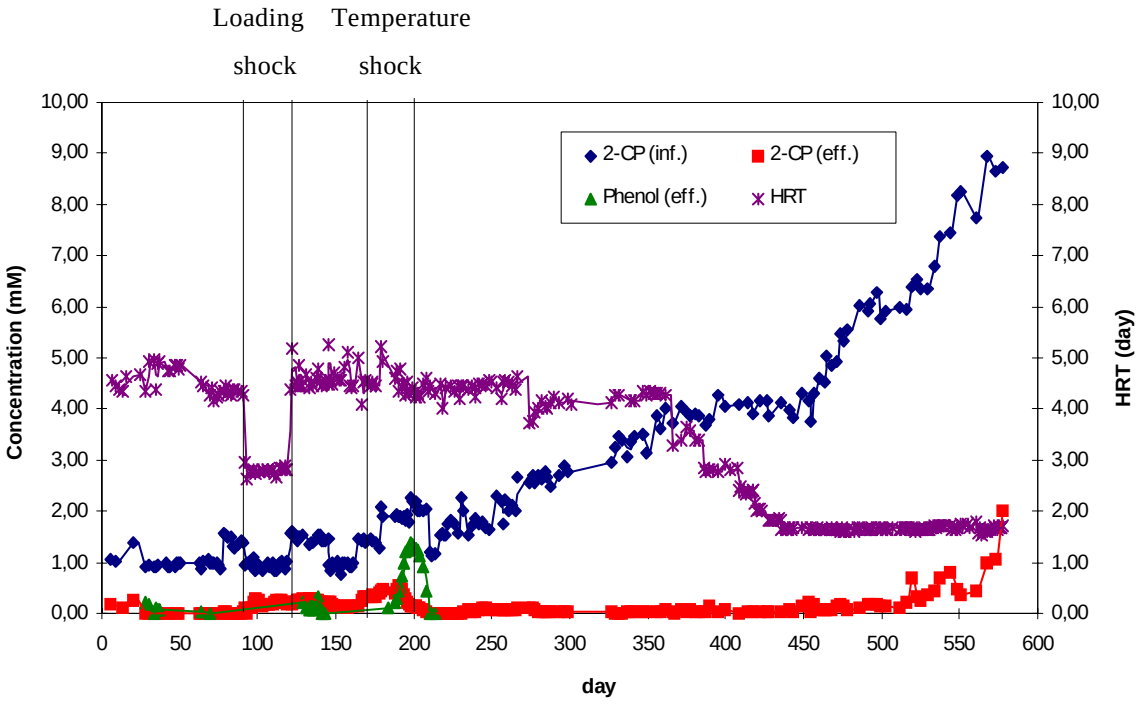


Fig. 3.11 a. Performance of reactor R 2 : Phenol, 2-CP concentration and HRT.

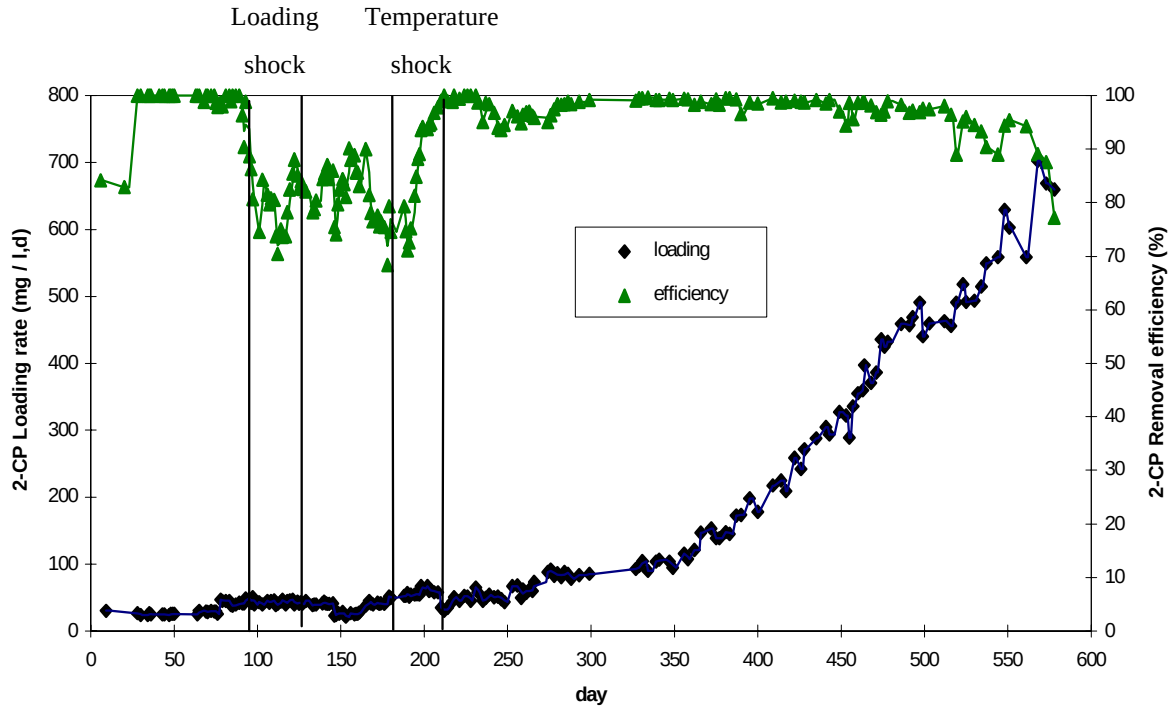


Fig. 3.11 b. Performance of reactor R 2 : 2-CP loading rate and % removal.

bioactivity the 2-CP degrading granules especially the COD removal rate. As illustrated in Figure 3.11 (b, c, d), the COD removal efficiency dropped readily from 85 to 22 % whereas the chlorophenol removal efficiency was only reduced by 20 %. The biogas production rate was reduced by more than 50% and the proportion of methane in biogas dropped from 60 to 32 %. The concentration of 2-CP in the effluent was increased and coupled with the appearance of phenol which was later degraded when the system was recovering. It took about 40 days for the 2-CP concentration in effluent, COD removal efficiency and biogas production rate to recover to the levels before the temperature shock.

3.4.3 COD removal, 2-CP removal and biogas production

After a full recovery of bioactivity of the pellets was accomplished, the 2-CP loading rate was gradually increased by either an increasing concentration of 2-CP in the influent or the reduction of the HRT (Fig. 3.11 a). A removal efficiency of 2-CP over 95 % was achieved for nearly 10 months during days 220-510 while the 2-CP loading rate was steadily raised from 50 to 500 mg/l, d (Fig. 3.11 b). A further increase of the 2-CP loading rate reduced the chlorophenol removal efficiency of the system and the 2-CP concentration in the effluent slowly increased. However, no trace of phenol was found in the effluent during this time. At the termination of the experiment the 2-CP loading rate was 700 mg/l, d, and the removal efficiency dropped to 76 %. 2-CP in the effluent had reached the concentration of 2 mM.

Since the concentration of glucose in the influent was kept constant at 0.9 g/l throughout the experiment, at a constant HRT the increasing of the organic loading rate was achieved by increasing the 2-CP concentration in the influent. As illustrated in Figure 3.11 c., the COD removal efficiency of the system after the temperature shock proceeded in the same manner as the 2-CP removal efficiency. An average COD removal efficiency of 85 % was attained during days 220-510 while the organic loading rate was increased from 0.38 to 1.60 g COD/l, d. The COD removal efficiency was steadily decreasing from 85 % to 70 % when the organic loading rate was further increased.

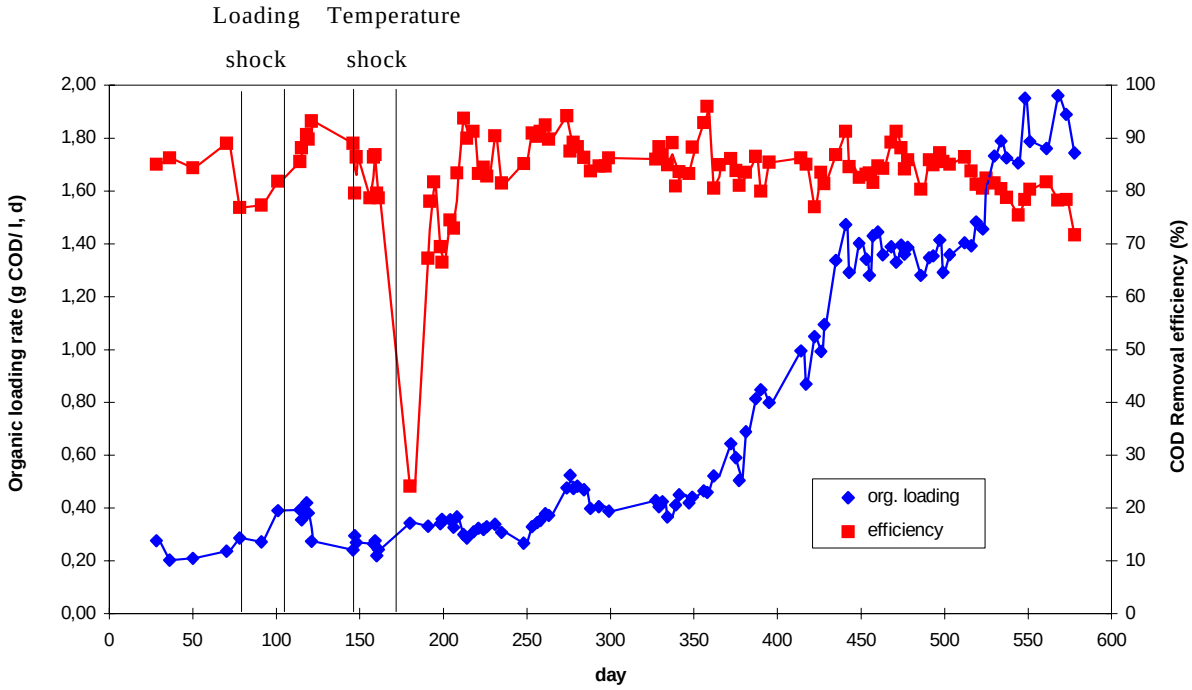


Fig. 3.11 c. Performance of reactor R 2 : COD load and % COD removal.

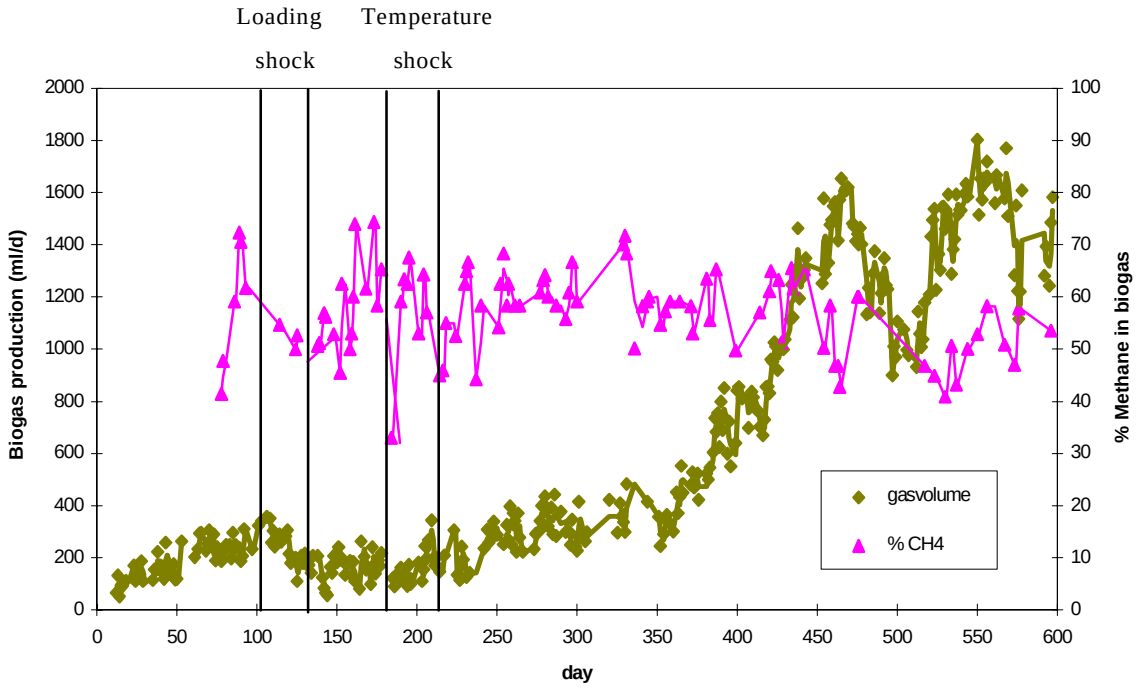


Fig. 3.11 d. Performance of reactor R 2 : Total gas production and % methane in biogas.

From start up until day 200 where the 2-CP loading rate was ≤ 50 mg/l, d, most of the biogas produced came from the degradation of glucose. After the system had recovered from the temperature shock, the total gas production increased along with the increasing glucose and 2-CP loading rate and reached 1.6 l/d on day 470 (Fig. 3.11 d). During days 470-500, biogas production decreased from 1.6 to 1.0 l/d and then increased again to

the preceding level on day 530. A decrease of total gas production can be seen again at the end of the experiment where the 2-CP loading rate was over 600 mg/l, d. A biogas production of about 200-300 ml less than the theoretically expected value (biogas production from glucose + 2-CP) at each loading rate was observed, presumably due to incomplete degradation of 2-CP to CH_4 and CO_2. The proportion of methane in the biogas, was between 50 and 70%. A lower methane content than 50% indicated a disturbance of methanogenesis in the reactor.

3.4.4 Chloride ion production

The net chloride production in the reactor was measured beginning with day 190 (after recovery from the temperature shock) to confirm the dechlorination of 2-CP. The results are presented in Figure 3.12. The maximum possible chloride ion production (drawn as a solid line in Figure 3.12) was calculated from the difference between the 2-CP concentration in influent and effluent, assuming reductive dechlorination with chloride ion release. A corresponding amount of chloride ions in the effluent to the theoretically expected maximum value was observed throughout the experiment, indicating that the disappearance of 2-CP in the reactor could be attributed to reductive dechlorination. At the termination of the experiment, the chloride concentration in the effluent had reached 350 mg/d which corresponded to 9.86 mM/d or 1267 mg/d of 2-CP that was dechlorinated.

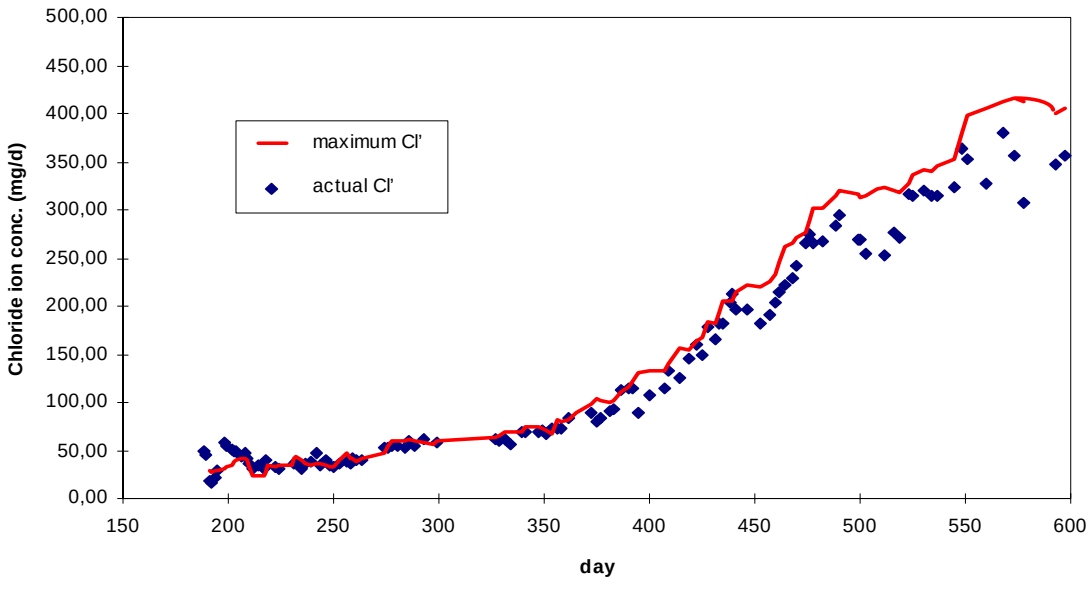


Fig 3.12 Actual and maximum net chloride ions production from the transformation of 2-CP in reactor R 2.

3.5 Mineralization and dechlorination rate of 2-CP in batch culture

Granular sludge from reactor R 2 (taken at day 90 after reactor start up) was incubated in serum bottles in the presence of 1 mM 2-CP +/- 5 mM glucose as a co-substrate. The initial dechlorination rate of glucose-amended cultures was about 43 mg/l,·d and phenol, the primary product from reductive dechlorination, was degraded within 4-5 days. When the 2-CP was completely mineralized, the same amount of 2-CP +/- glucose was refeeded. After 4 times of refeeding, the dechlorination rate of glucose-amended cultures had improved to approximately 64 mg/l,·d but the removal of phenol still required the same time (Fig. 3.13 a). The addition of 5 mM glucose after 2-CP was completely dechlorinated had no significant effect on the phenol degradation rate of this culture.

The initial dechlorination rate of non-glucose amended cultures was only 22 mg/l,·d but the phenol removal rate was the same as in glucose-amended cultures (Fig. 3.13 b). Refeeding

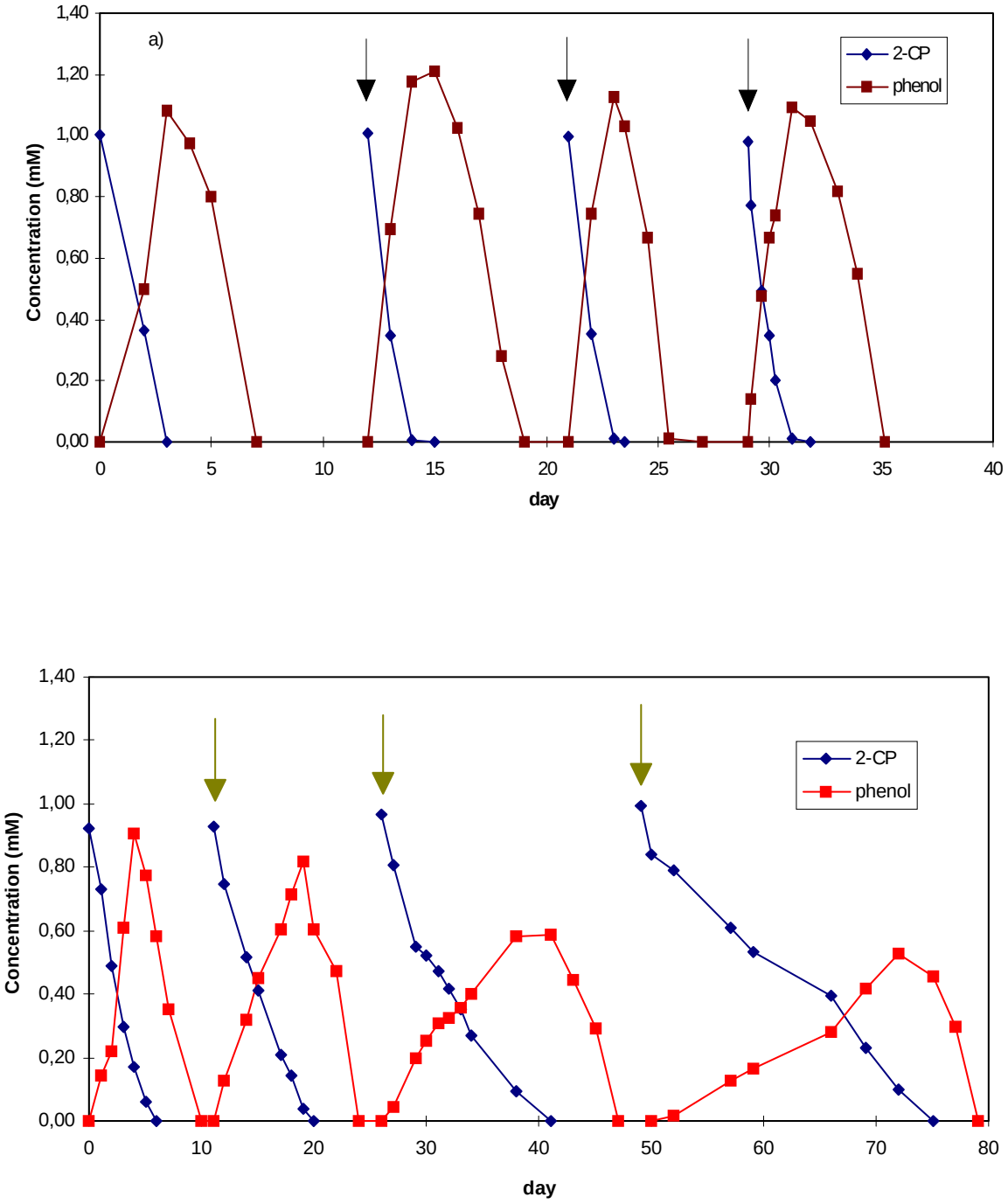


Fig. 3.13 Degradation of 2-CP and phenol in the cultures amended with glucose as co-substrate (a) and in non-glucose amended cultures (b).

of 2-CP could improve neither dechlorination nor the mineralization rate of this culture. On the other hand, the dechlorination rate was gradually decreasing from 22 mg/l,·d (1st addition) to 5 mg/l,·d (4 th addition). Whereas 1 mM 2-CP was completely degraded to methane and CO_2 in the glucose-amended batch cultures within 5-7 days in the non-glucose-amended cultures complete degradation required 10-20 days.

Theoretically 1 mM of 2-CP was dechlorinated to 1 mM of phenol (Eq. 2) and when 2-CP finally was completely mineralized, 3.25 mM of CH_4 should be obtained (Eq. 3).

$$C_6H_4OHCl + H_2 \longrightarrow C_6H_5OH + HCl \quad \text{.....(2)}$$

$$C_6H_4OHCl + 4.5\ H_2O \longrightarrow 2.75\ CO_2 + 3.25\ CH_4 + HCl \quad \text{.....(3)}$$

The amount of methane in the first 9 days of the experiment, in which only 2-CP in glucose-amended cultures was completely degraded, corresponded to the stoichiometric value expected for complete degradation of 2-CP (Fig. 3.14). The formation of methane in both cultures indicated that dechlorination did not inhibit methanogenesis from glucose.

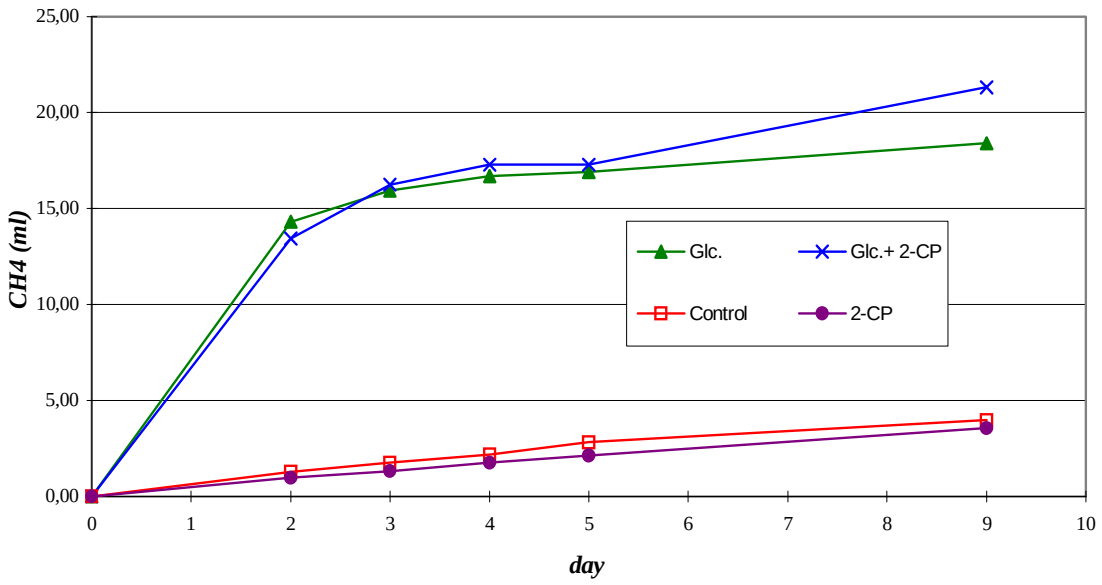


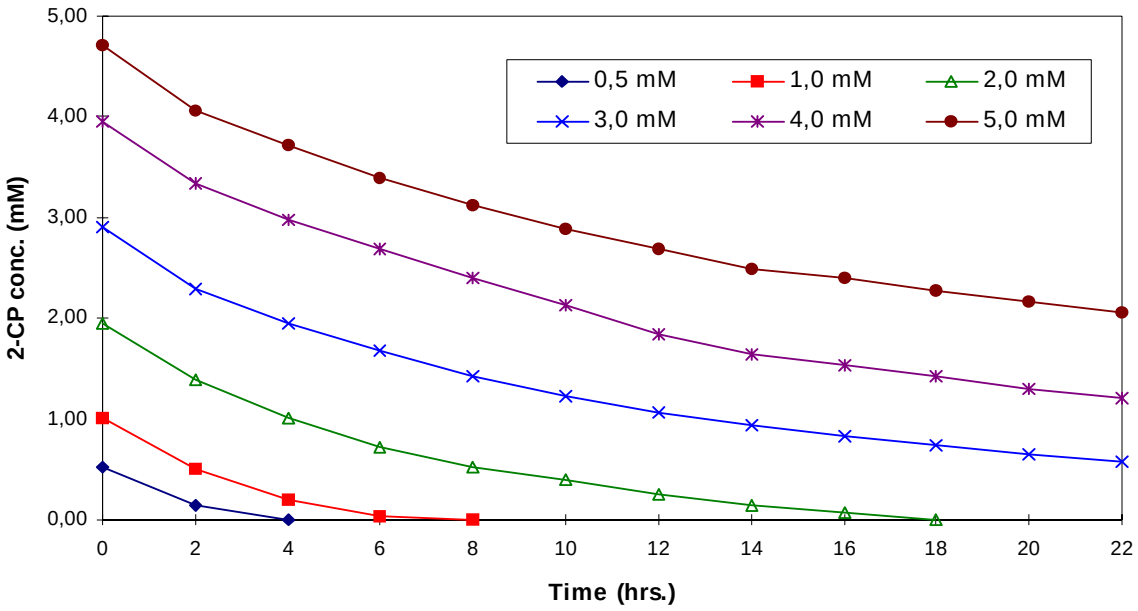
Fig. 3.14 Methane produced in glucose-amended cultures and non-glucose amended cultures in the presence of 2-CP. Control = background methane formation of this culture

3.6 Dechlorination kinetics

2-CP degrading granules were taken from reactor R 2 at a 2-CP loading of between 51 and 116 mg/l, d (day 220-360 of reactor operation) to determine the specific dechlorination rate and kinetic parameters of the dechlorination. Concentrations of 2-CP used were between 0.5-5 mM and a fixed glucose concentration of 0.9 g/l (5 mM) served as co-substrate in each sample. The depletion curve of 2-CP and the amount of phenol, the first intermediate of the dechlorination, from one of the experiments are demonstrated in Figure 3.15 and 3.16. It can be seen that the dechlorination rate was drastically increased (7-9 times) compared to the rate at the start of the reactor (see 3.5). This suggests a good adaptation of this biomass to 2-CP.

Fig. 3.15 Biotransformation of different concentrations of 2-CP.

Figure 3.17 shows the results of the kinetic experiment. The dechlorination rates were calculated as the



slope of the regression line of the 2-CP depletion curve. Each initial concentration of 2-CP was the actual concentration measured at the begining of the experiment (t = 0). Determination of the half velocity constant (K_m)and the maximum dechlorination rate (V_{max}) using a graphical method according to Lineweaver - Burk gave a K_m value of 1.18 mM (= 152 mg/l) and a V_{max} of 66.4 mg/g TS, d.

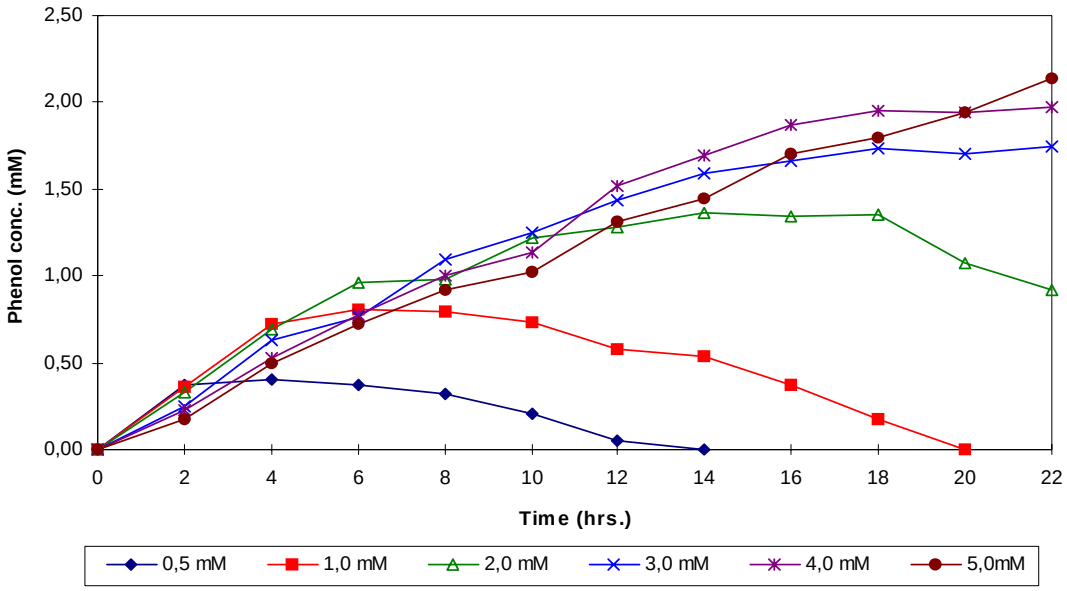


Fig. 3.16 Amount of phenol that occured during dechlorination of various concentrations of 2-CP.

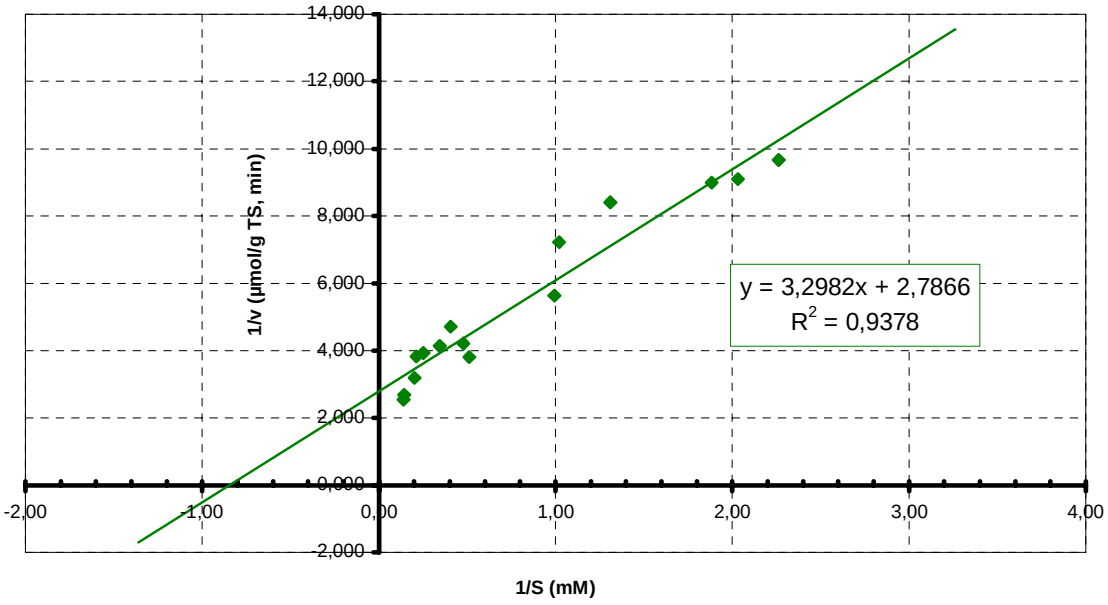


Fig. 3.17 Lineweaver-Burk plot to determine the maximum dechlorination rate V_{max} and the half velocity constant, K_m.

With respect to V_{max} , a 2-CP loading rate of 511 mg/l, d (based on a total biomass concentration of 20 g) was estimated as the maximum dechlorination rate for this system. Therefore, the 2-CP loading rate of reactor R 2 was stepwise increased by decreasing the HRT or increasing the 2-CP concentration in the influent (starting

from day 360 on). As shown in Figure 3.11 b., a 2-CP loading rate of more than 600 mg/l, d in the last phase of operation verified the validity of this estimation.

On day 462 of the reactor operation, biomass was taken to determine the specific dechlorination rate again. It was found that the initial dechlorination rate of 2-CP for a concentration range from 0.5-5 mM 2-CP in each sample was in the same range between 0.07-0.08 mM/h (Fig. 3.18).

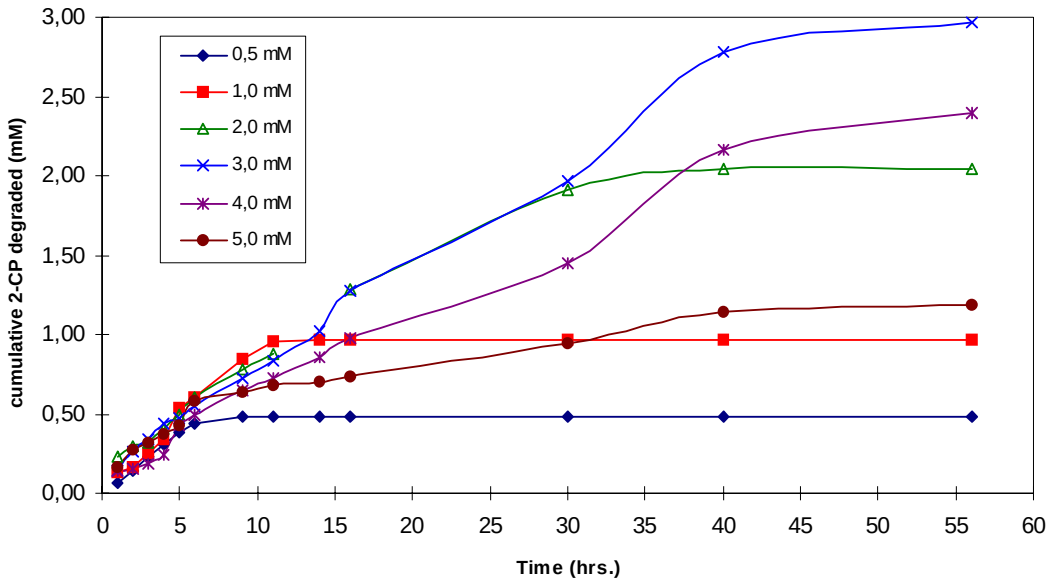


Fig. 3.18 Dechlorination of 2-CP at various initial concentrations of 2-CP.

3.7 Effect of glucose on dechlorination at higher 2-CP concentration

Reactor R 2 was operated for more than 350 days with increasing 2-CP concentration. A chlorophenol removal efficiency over 95 % was attained at a fixed glucose concentration of 0.9 g/l. This indicated the potential ability of these sludge granules to use 2-CP as another C-source. Moreover, it can be seen from Figure 3.15 that 2-CP in samples which have concentrations ≤ 2 mM was removed within 24 hours in the presence of 5 mM glucose. Thus, it should be examined whether glucose still enhanced the dechlorination by this biomass at 2-CP concentration higher than 2 mM.

The 2-CP degrading granules were incubated with 3, 4 and 5 mM 2-CP in the presence/absence of 5 mM glucose. The repeated addition of the same concentrations of glucose was performed in one of the glucose-amended samples when the initial amount of glucose was exhausted. The concentration of 2-CP and phenol in each sample is shown in Figure 3.19 (a-c). The acceleration of dechlorination by repeating glucose additions was markedly seen only in samples which were incubated with 4 mM of 2-CP (Fig. 3.19 b). In these samples, 2-CP

was almost completely dechlorinated in 80 hours while it took more than 120 hours in one-time glucose amended and non-amended samples. The dechlorination and phenol transformation rates of glucose-amended and non-glucose amended samples for 2-CP concentrations of 3 mM and 5 mM were not significantly different. Furthermore, repeated addition of glucose neither did enhance the dechlorination rate nor the phenol transformation rate, as can be obviously seen in samples incubated with 5 mM of 2-CP (Fig. 3.19 b). Despite 6 times of glucose refeeding within 5 weeks, the same amount of 2-CP (ca. 2 mM) was dechlorinated like in others samples. Results showed that glucose was still required by the adapted biomass for dechlorination, although no marked effect of glucose addition was observed in samples incubated with 3 and 5 mM of 2-CP. Therefore, 5 mM of glucose was further fed as co-substrate in the influent for reactor R 2 in order to ensure and enhance the dechlorination of 2-CP in the system.

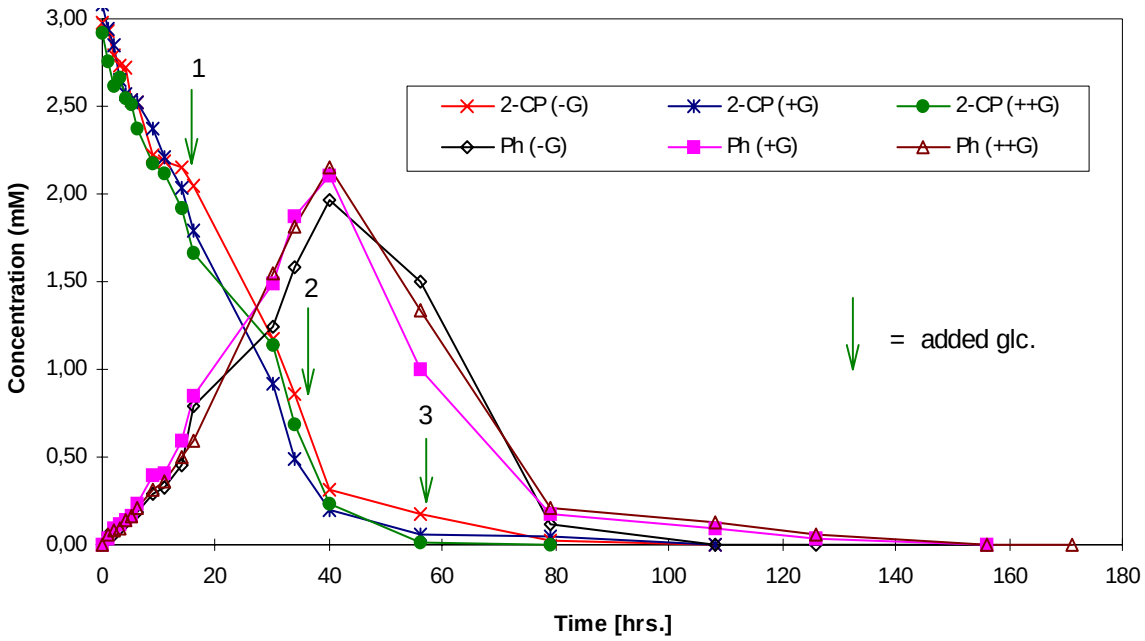


Fig. 3.19 a. 2-CP and phenol concentration in samples which were incubated with 3 mM of 2-CP an a different regime of glucose addition. Abbreviation : -G = no glucose addition, +G = one-time glucose addition, ++G = more times glucose addition.

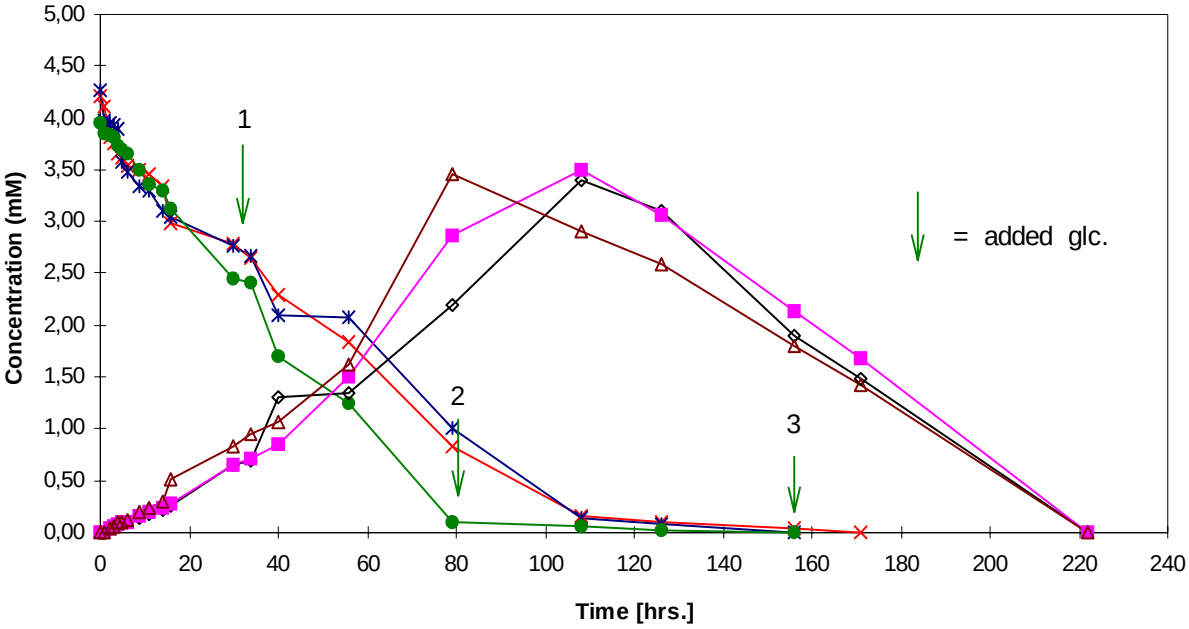


Fig. 3.19 b. 2-CP and phenol concentration in samples which were incubated with 4 mM of 2-CP and a different regime of glucose addition. Abbreviations and symbols are the same as in Fig. 4.19 a.

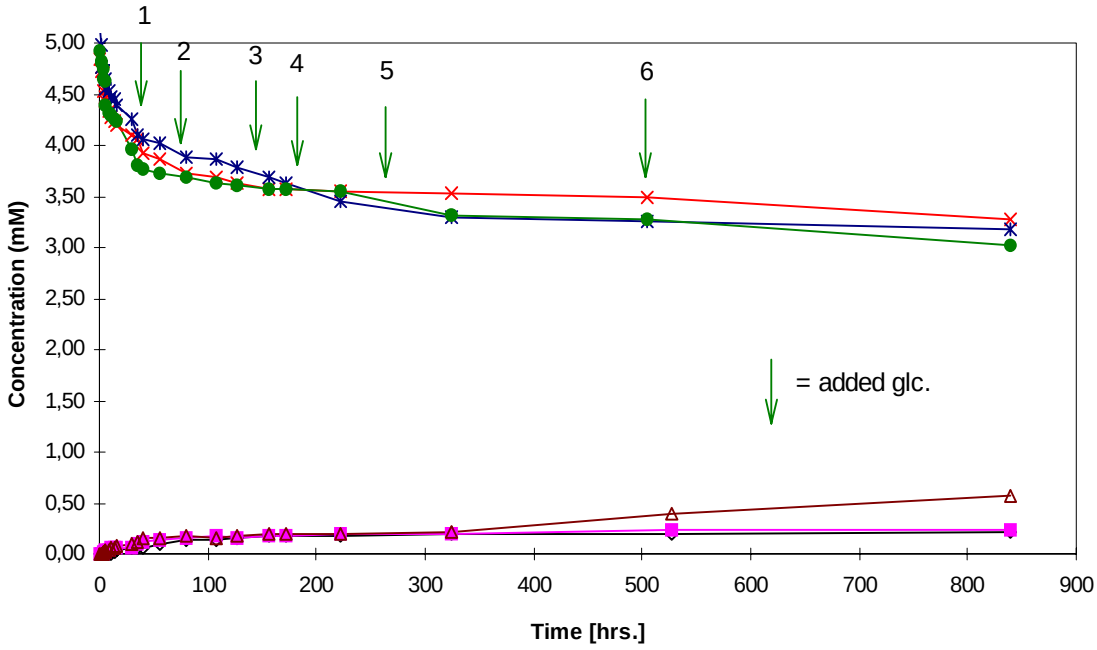
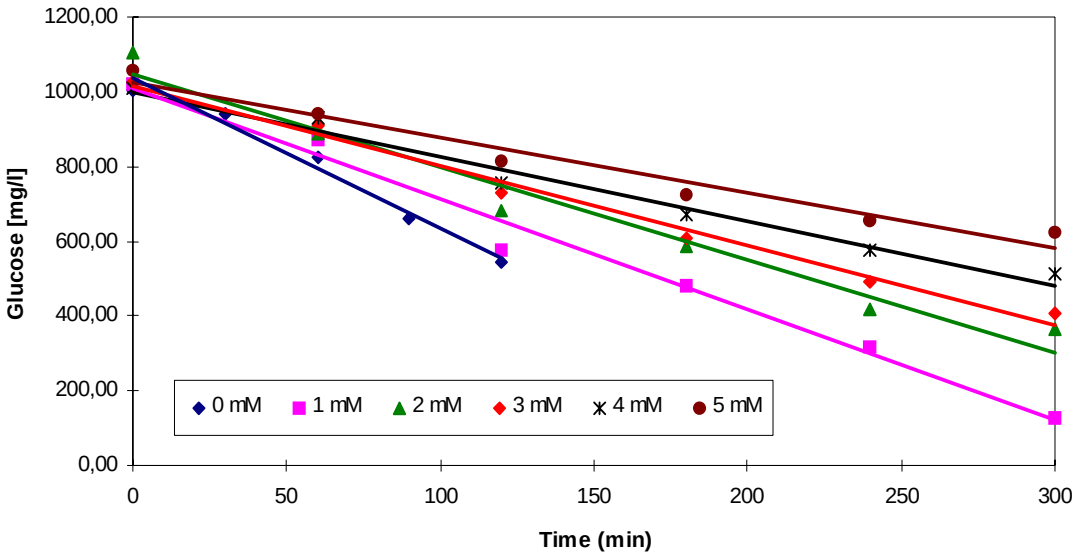


Fig. 3.19 c. 2-CP and phenol concentration in samples which were incubated with 5 mM of 2-CP and a different regime of glucose addition. Abbreviations and symbols are the same as in Fig. 4.19 a.

3.8 Kinetics of glucose degradation

Glucose was used as a co-substrate for treatment of a 2-CP containing synthetic wastewater in a UASB reactor to ensure the dechlorination evtl. degradation of 2-CP. Hence, the impact of 2-CP on kinetic parameters of glucose degradation by 2-CP degrading granules (adapted biomass) in comparison with glucose-degrading granules (non-adapted biomass) should be investigated.

Figure 3.20 illustrates the inhibition effect of 2-CP on glucose degradation of non-adapted biomass at a glucose concentration of 1000 mg/l. The glucose degradation rate (calculated from the slope of each curve) decreased with an increasing concentration of 2-CP. Figures 3.21 and 3.22 show the Dixon plot (a) and Cornish-Bowden plot (b) in the presence of different fixed glucose concentrations of non-adapted biomass and of biomass



that was adapted to 2-CP, respectively.

Fig. 3.20 Degradation of 1000 mg/l glucose by non-adapted (unacclimated) biomass which was incubated with various concentrations of 2-CP.

From the Dixon and Cornish-Bowden plot, it was concluded that 2-CP was an inhibitor of glucose degradation for both adapted and non-adapted biomass. The type of inhibition was a mixed inhibition in which for adapted biomass the K_i value (dissociation constant of the EI complex) was higher than K_{ii} (dissociation constant of the ESI complex) and vice versa for non-adapted biomass. The kinetic parameters for glucose degradation, estimated by the graphical method, are presented in Table 3.2.

Table 3.2 : Kinetic parameters for glucose degradation by non-adapted and adapted biomass in the presence of 2-CP concentrations ranging from 0 to 5 mM.

kinetic parameters	**V_{max}** (g glc./gTS, d)	**K_m** (mg glc./l)	**K_i** (mg 2-CP/l)	**K_{ii}** (mg 2-CP/l)
non-adapted biomass	1.1486	22.21	116.27	501.32
adapted biomass	2.0065	322.07	705.72	152.37

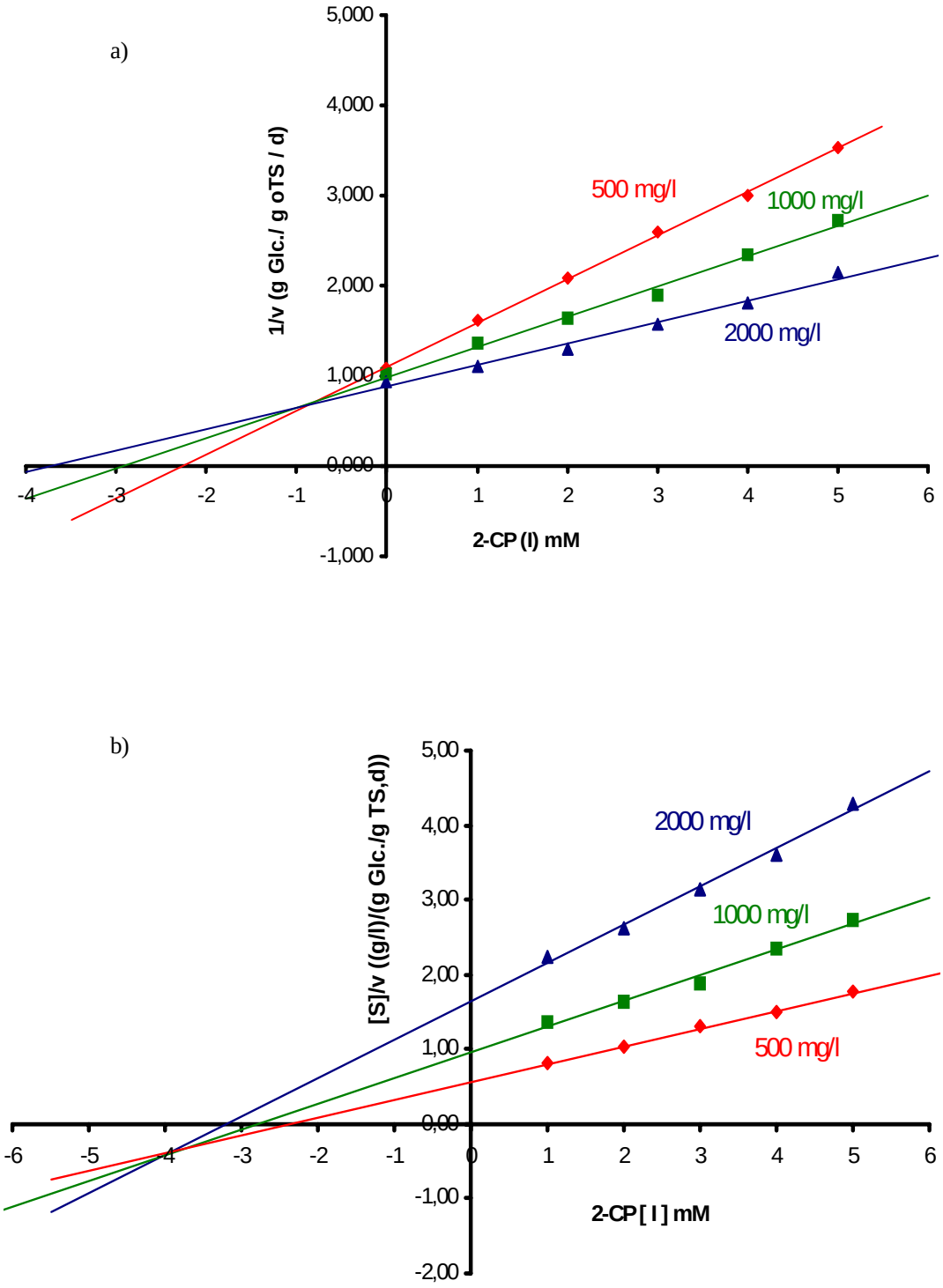


Fig. 3.21 Dixon (a) and Cornish-Bowden plot (b) of non-adapted biomass in the presence of 5 different concentrations of 2-CP and three different concentrations of glucose.

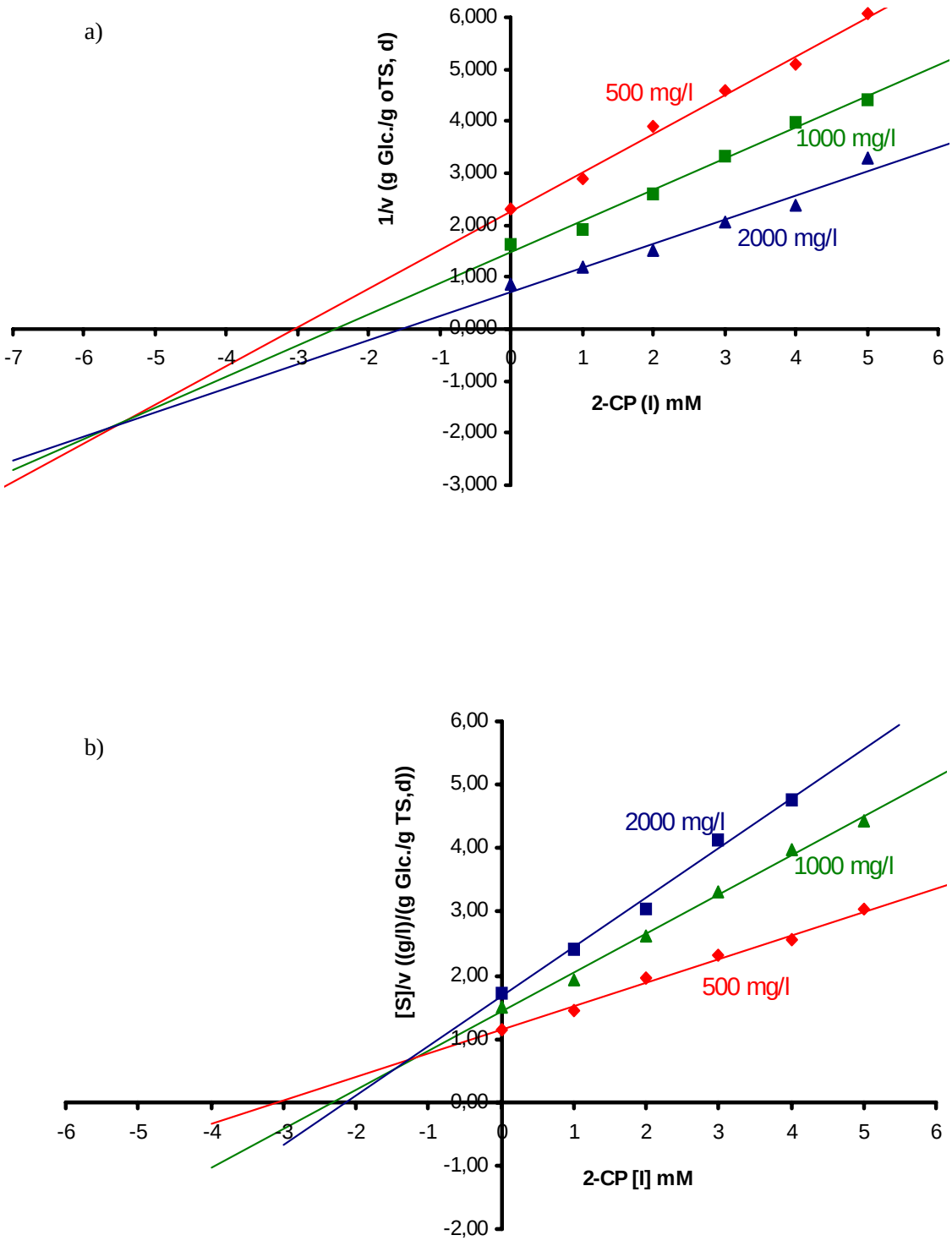


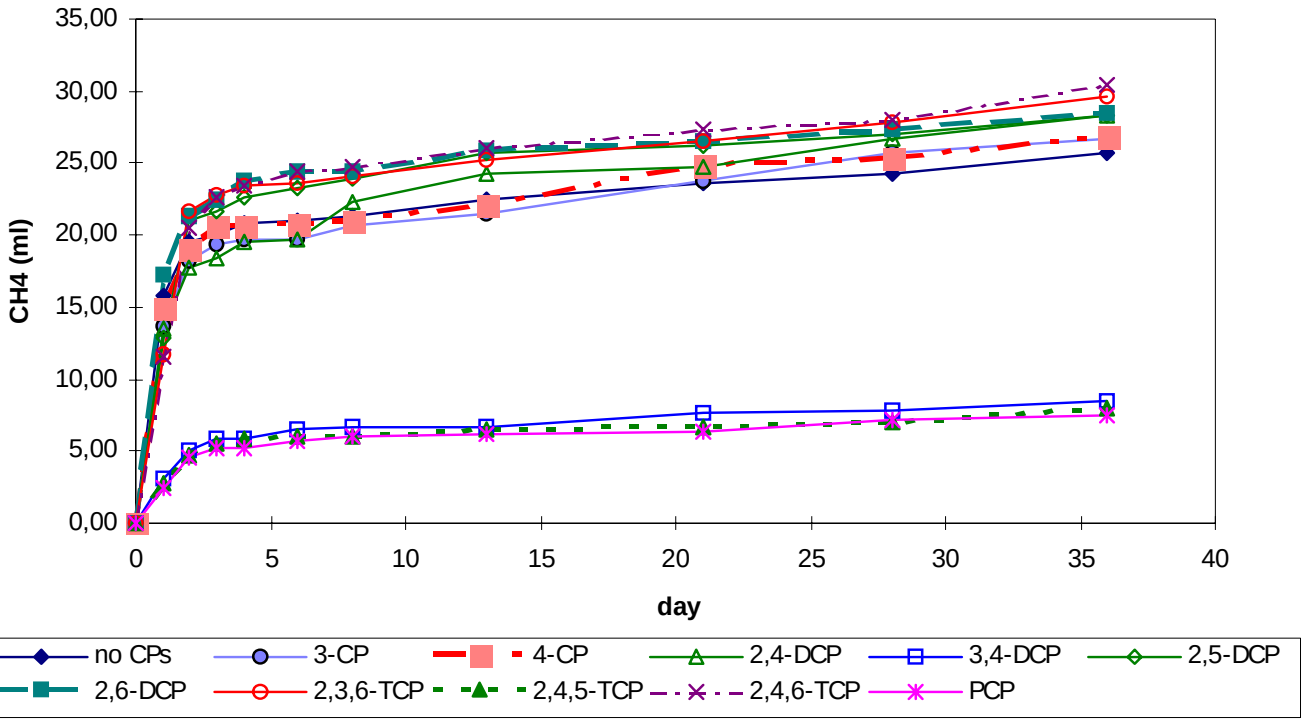
Fig. 3.22 Dixon (a) and Cornish-Bowden plot (b) of adapted biomass in the presence of 5 different concentrations of (2-CP) and three different concentrations of glucose.

3.9 Degradation potential of 2-CP-degrading granules for other chlorophenols

3.9.1 Glucose as electron donor

The 2-CP degrading granules from reactor R 2 were incubated with 0.5 mM of 10 different chlorophenols (3-CP, 4-CP, 2,4-DCP, 2,5-DCP, 2,6-DCP, 3,4-DCP, 2,3,6-TCP, 2,4,5-TCP, 2,4,6-TCP, and PCP) and 5 mM of glucose. The amount of methane gas produced in each sample over 35 days of incubation is shown in Figure 3.23. Figure 3.24-3.26 show the concentration of parental mono-, di-, tri- or pentachlorosubstituted phenols evtl. the intermediates and the net chloride ion concentration in each sample.

Fig. 3.23 Amount of methane produced in the samples which were incubated with different chlorophenols



The 2-CP degrading granules were able to completely degrade 4-CP within 13 days whereas the transformation of 3-CP took longer time with a little initial lag period (Fig. 3.24). The amount of chloride ions released from transformation of both MCPs corresponded with the quantity of biotransformed chlorophenols. No phenol was detected during transformation of 3-CP and 4-CP by this culture, indicating that phenol was not the rate-limiting step and presumably was further degraded to methane and CO_2 .

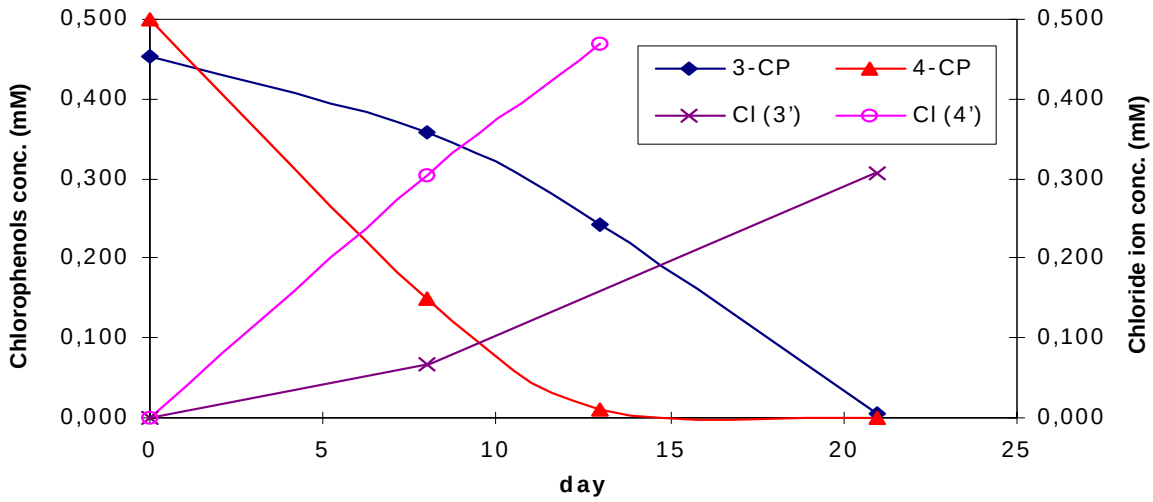


Fig. 3.24 Degradation of 3-CP, 4-CP and amount of chloride ions released during dechlorination by 2-CP degrading cultures.

Except for 3,4-DCP all tested dichlorophenols were completely degraded in a period of time, ranging from less than 2 days to 40 days (Fig. 3.25). The transformation of 3,4-DCP occured mainly in the first few days of incubation but about half of the initial concentration remained in the sample. Two monochlorophenols, 3-CP and 4-CP, were found as intermediates from the dechlorination of 3,4-DCP but in little less than stoichiometric amounts (Fig. 3.25 b). 2,4-DCP and 2,5-DCP were first dechlorinated to 4-CP and 3-CP, and were then completely transformed in 15 and 36 days, respectively (Fig 3.25 a, c). Total disappearance of 2,6-DCP occured in less than 2 days and no other chlorophenols or phenol was detected in this sample (Fig. 3.25 d). An amount of chloride ions corresponding to the amount of transformed dichlorophenol was found in each tested sample.

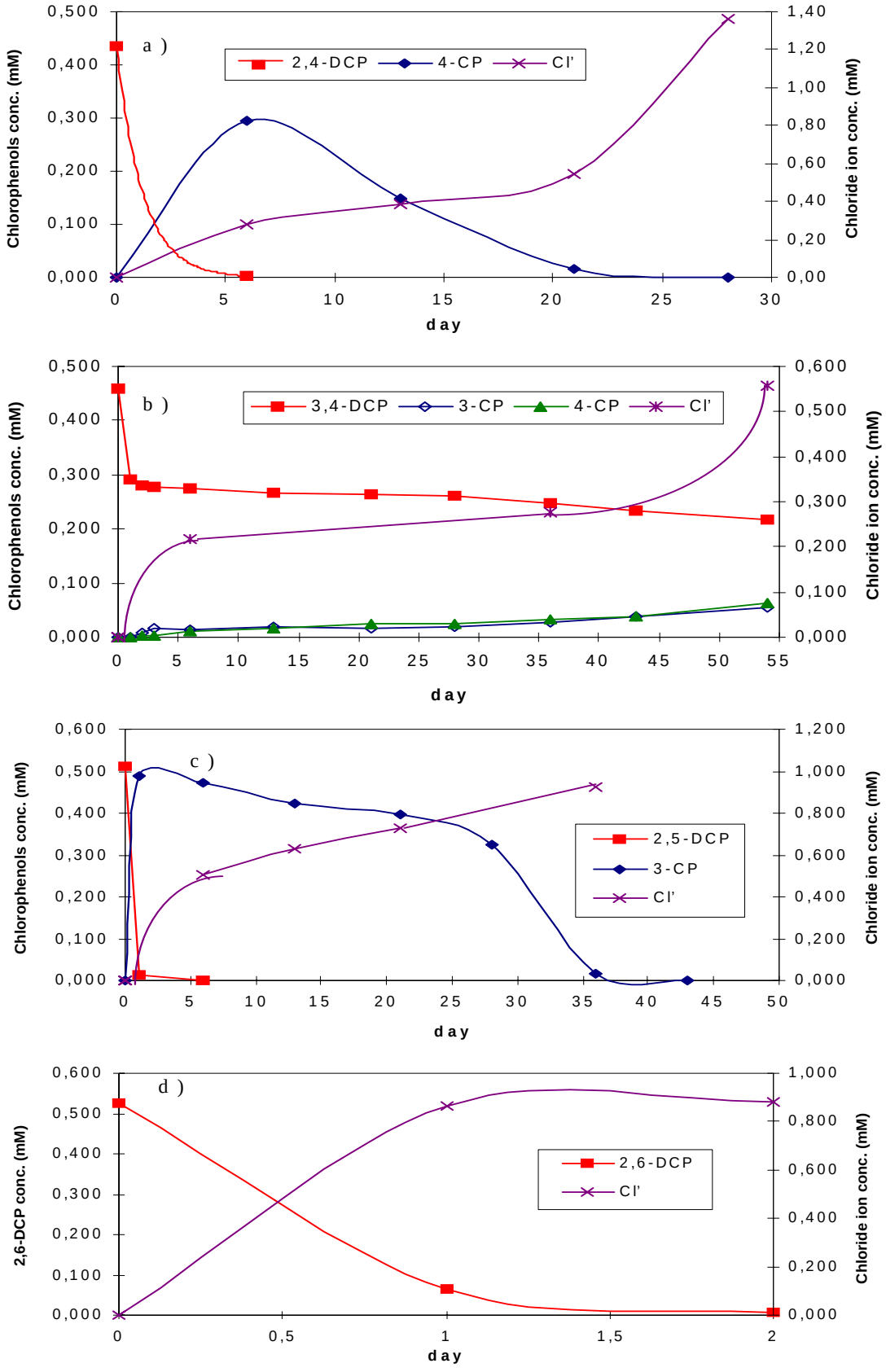


Fig. 3.25 Degradation of (a) 2,4-DCP (b) 3,4-DCP (c) 2,5-DCP (d) 2,6-DCP and amount of chloride ions released from the dechlorination by 2-CP degrading cultures.

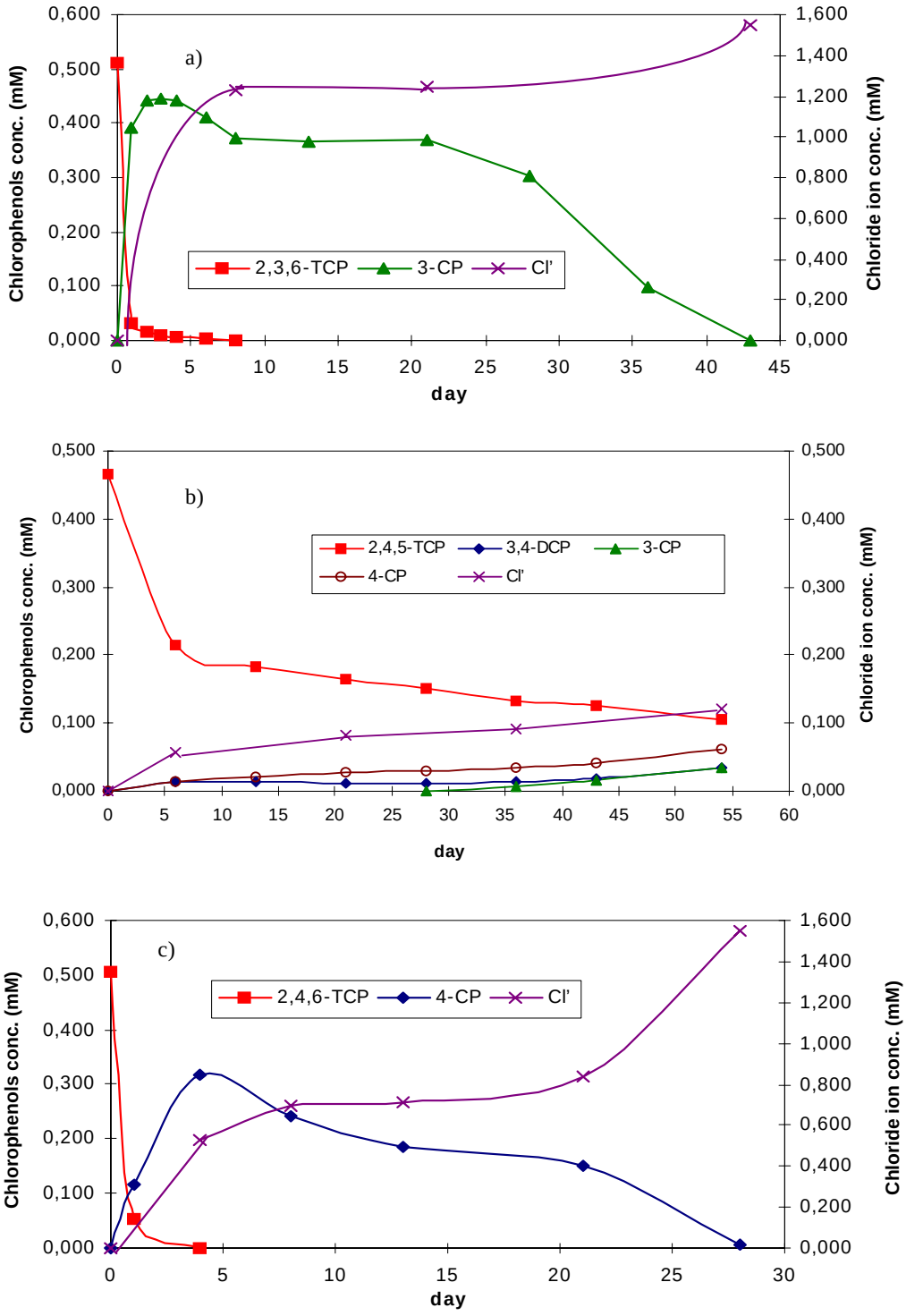


Fig. 3.26 Degradation of (a) 2,3,6-TCP (b) 2,4,5-TCP (c) 2,4,6-TCP and amount of chloride ions released from the dechlorination by 2-CP degrading cultures.

The complete transformation of trichlorophenol by 2-CP degrading granules was observed in samples which were incubated with 2,3,6-TCP and 2,4,6-TCP. Both trichlorophenols were double dechlorinated to 3-CP and 4-CP, respectively, without the appearance of any DCP as intermediate (Fig 3.26 a, c). Moreover, the stoichiometric amount of chloride ions in both samples was also obtained. The sequential dechlorination was observed from the transformation of 2,4,5-TCP, in which 2,4,5-TCP was dechlorinated via 3,4-DCP to 3-CP and 4-CP (Fig. 3.26 b). However, the amount of intermediates was relatively low compared to amount of 2,4,5-TCP that disappeared.

Pentachlorophenol was mainly removed at the begining of the incubation by adsorption. After that the concentration of PCP was nearly unchanged (Fig. 3.27). Some TCP, DCP, MCP and phenol were found in the samples which were incubated with PCP, indicating that there was little biotransformation and not only adsorption to the sludge pellets. Due to the lack of some TCP references and due to the fact that all DCP have nearly the same retention time under the analytic conditions, the peak area of each substance was presented instead of a concentration to demonstrate the occurence of each intermediate in the samples (Figure 3.28). Trichlorophenol (2,4,5-TCP and an unknown isomer) was the major intermediate on day 13 but disappeared after that. On day 28, only MCP and phenol were observed in the samples. TCP (2,4,5- and 2,4,6-TCP) appeared again on day 43 while DCP was first detected on day 54 of the incubation. 3-CP, 4-CP and phenol were always found throughout the incubation period and had a trend to increase with the time.

Methane formation from glucose in samples which were incubated with 0.5 mM 3,4-DCP, 2,4,5-TCP and PCP was more than 80 % inhibited whereas in other samples the amount of methane corresponded to the amount of added glucose. Moreover, a higher methane amount than in control samples (granules incubated only with 5 mM glucose) was observed when the parent chlorophenols were completely removed (Fig. 3.23).

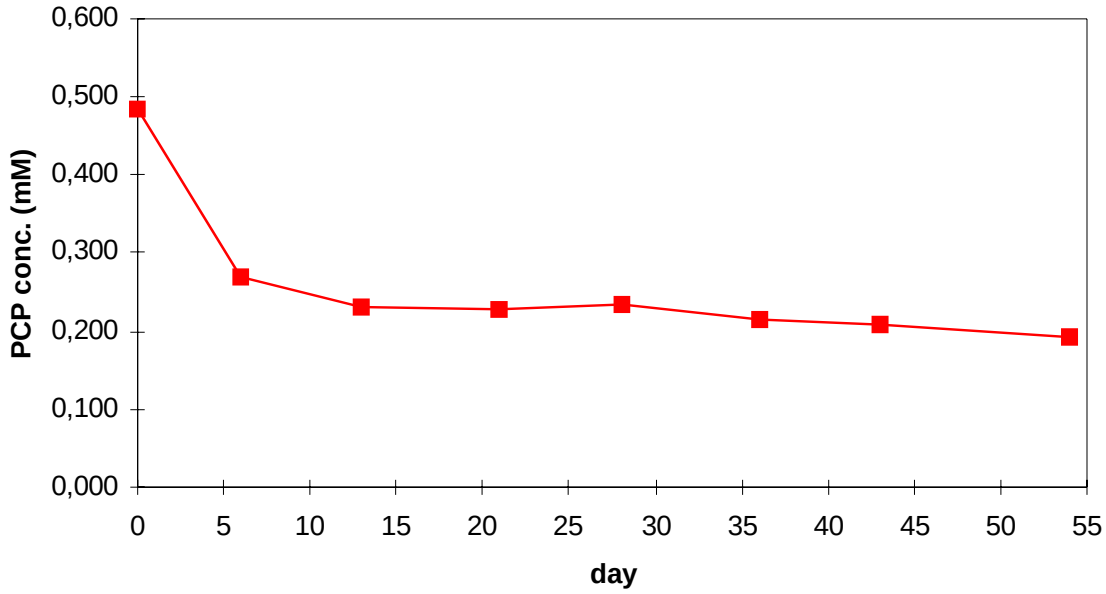


Fig. 3.27 Adsorption / transformation of PCP by 2-CP degrading cultures.

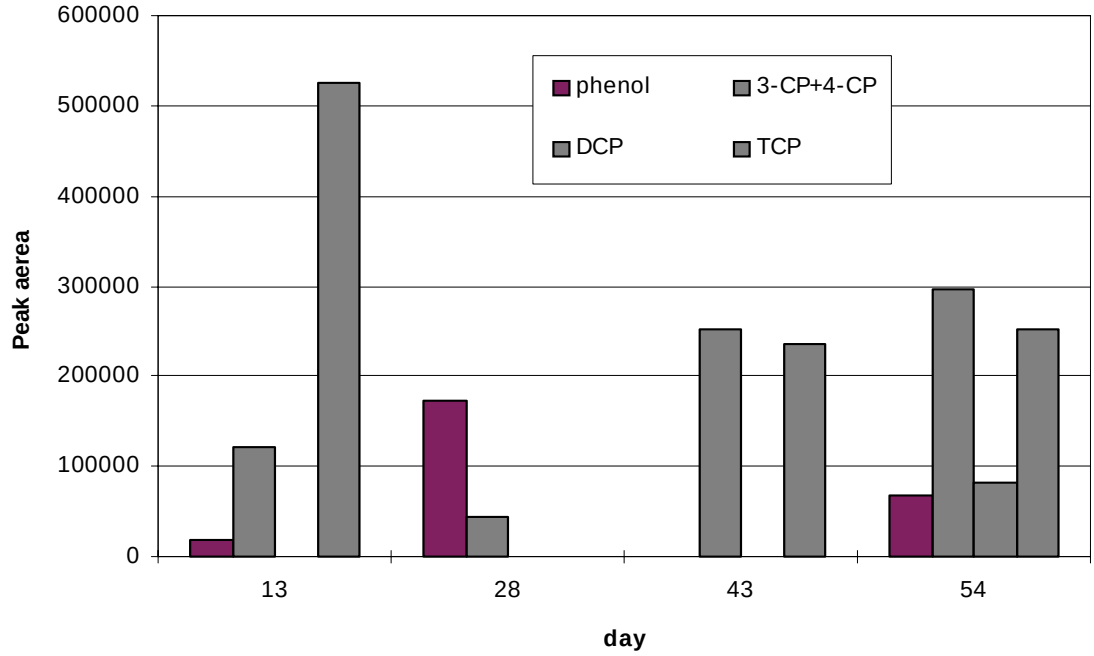
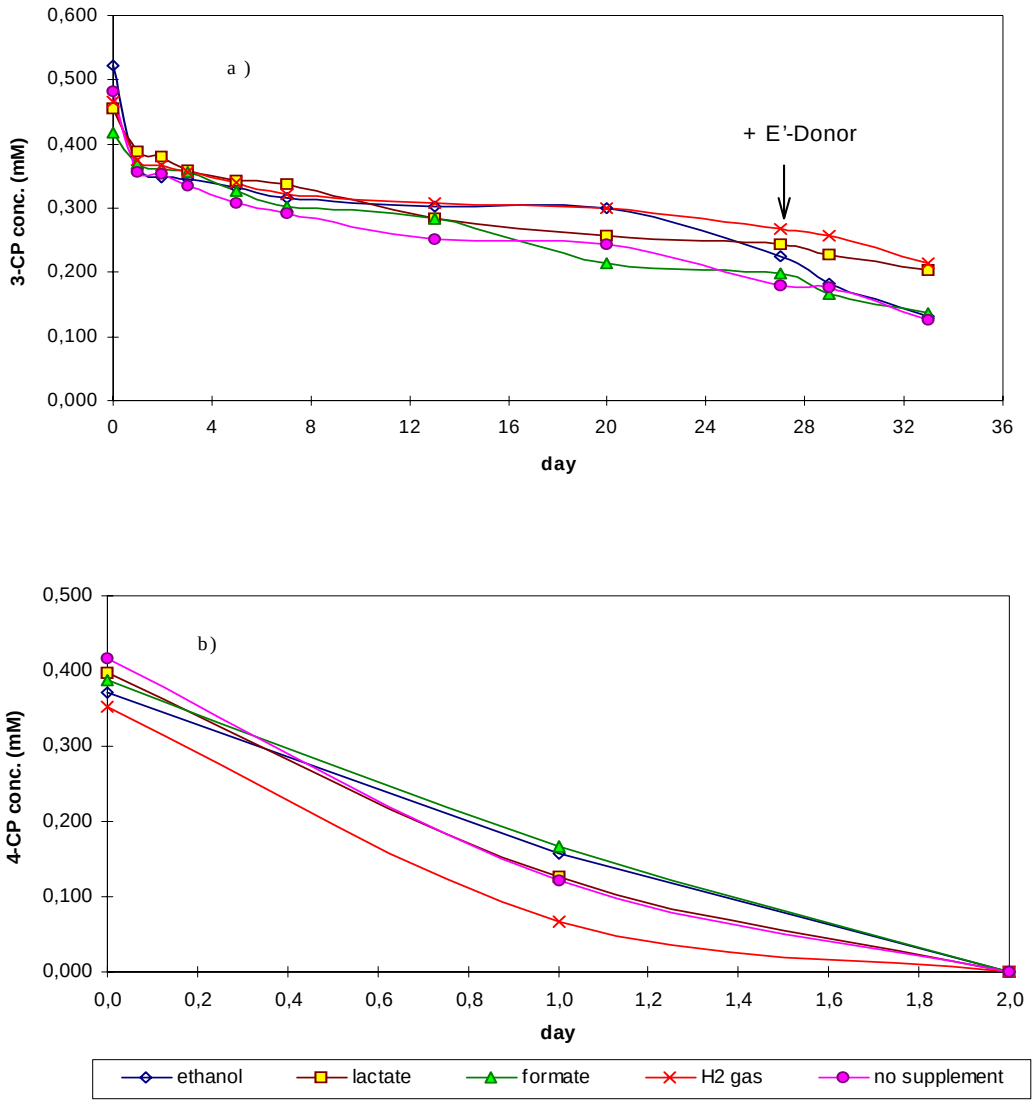


Fig. 3.28 Intermediates (expressed in form of peak area) occuring during the transformation of PCP by 2-CP-degrading cultures.

3.9.2 Other electron donors

Apart from glucose, other organic substrates (i.e. ethanol, lactate, formate) in concentration of 5 mM and 1 atm of H_2 gas were used as electron donors for evaluation of the potential of 2-CP degrading granules to transform other chlorophenols. A sample without any electron donor was also tested. Table 3.3 shows % transformation of each chlorophenol, incubated with various electron donors after 27 days of incubation.

Fig. 3.29 Transformation of 3-CP (a) and 4-CP (b) by 2-CP degrading cultures amended with various electron donors.



In the presence and absence of these electron donors, 3-CP was less transformed than when it was incubated with glucose (total dechlorination after 21 days) and it was not completely removed within 32 days of the incubation (Fig. 3.29 a). About 50 % of 3-CP was dechlorinated when ethanol, lactate, H_2 and formate were used as electron donors but little more 3-CP was transformed in non-amended cultures (see Table 3.3). In

contrast to 3-CP, total disappearance of 4-CP occured within 2 days with all electron donors and 4-CP was faster transformed when hydogen gas was used as electron donor (Fig. 3.29 b). Dechlorination of 2-CP and 4-CP of granules adapted to 2-CP occured at a similar rate, whereas 3-CP could only be dechlorinated at a much lower rate.

3,4-DCP was mainly removed at the begining of the incubation. Only 3-CP was found as the intermediate from the dechlorination of 3,4-DCP (Fig. 3.30 a, b), indicating that chlorine removal from the *meta*-position was much slower than from the *para*-position.

No complete degradation of 2,4-DCP, 2,5-DCP and 2,6-DCP was observed in this experiment. This may have been due to high initial concentrations of the parent substances (between 1.1-1.3 mM). The concentrations of these three dichlorophenols and of their intermediates are presented in Figure 3.31-3.33. The chlorine atom at the *ortho* position of 2,4-DCP, 2,5-DCP and 2,6-DCP was first dechlorinated and yielded 4-CP, 3-CP and 2-CP as intermediate, respectively. It was rather surprising that 2,6-DCP was the least transformed among these three DCPs, opposed to the rapid removal of this DCP isomers when incubated with glucose. In addition, 2-CP begun to accumulate in some samples after 8 days of incubation (Fig. 3.33 b). During 27 days of incubation, only the disappearance of 3-CP, an intermediate from 2,5-DCP transformation was observed (Fig. 3.32 b).

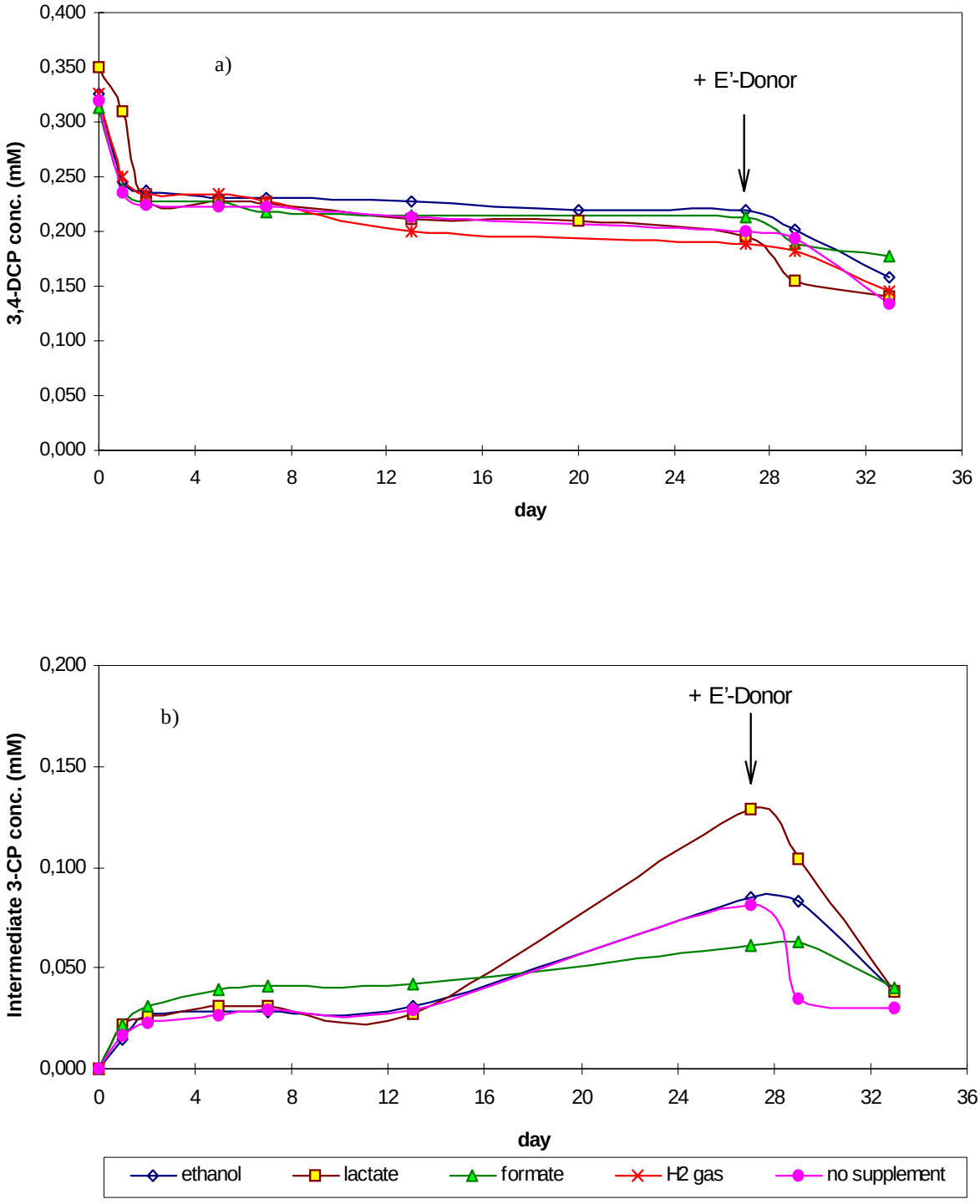


Fig. 3.30 Transformation of 3,4-CP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 3,4-DCP (b) concentration of 3-CP (intermediate from dechlorination of 3,4-DCP).

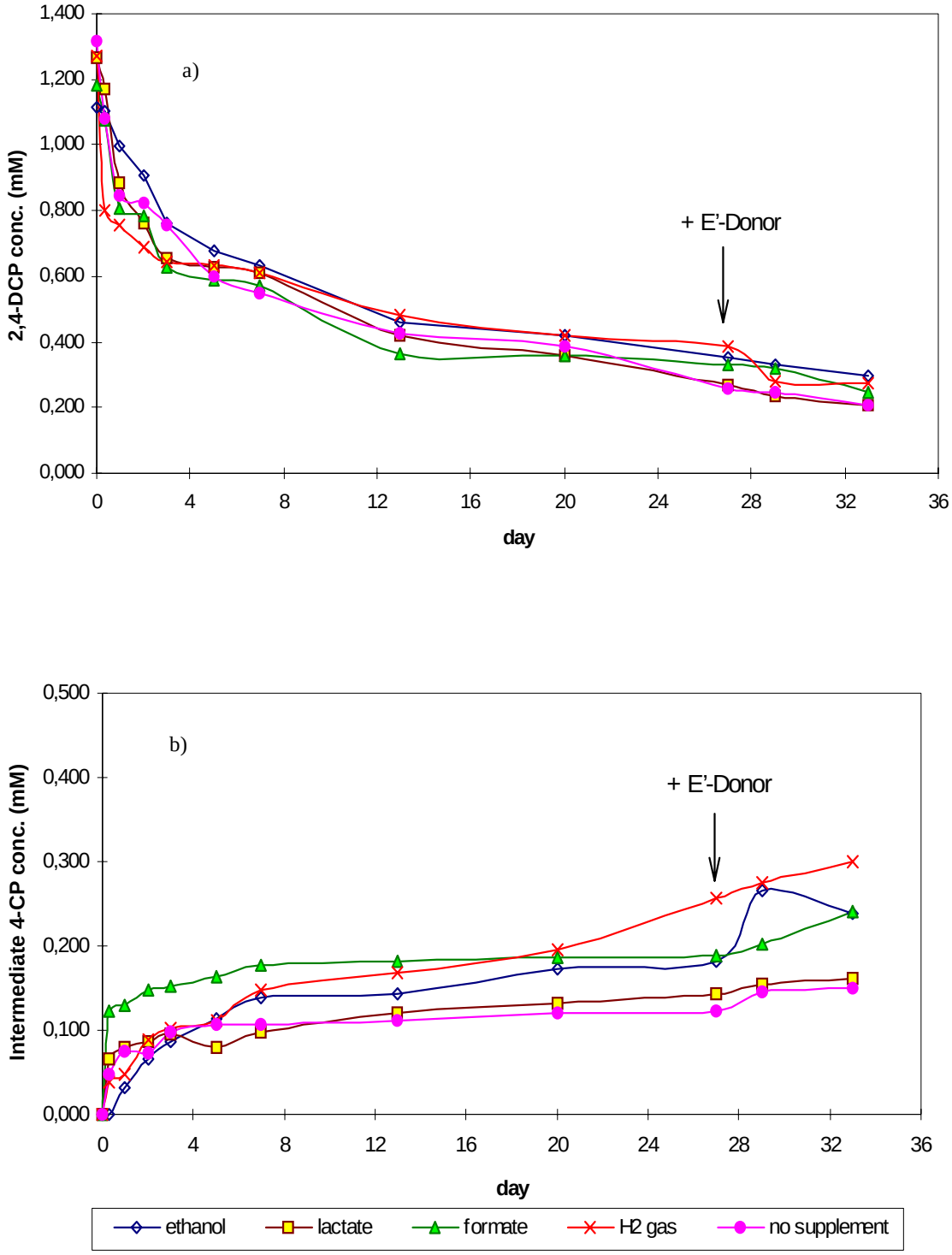


Fig.3.31 Transformation of 2,4-DCP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 2,4-DCP (b) concentration of 4-CP (intermediate from dechlorination of 2,4-DCP).

Table 3.3 Percentage transformation of chlorophenols incubated with various electron donors on day 27 of incubation.

Chlorophenol	% Transformation				
	ethanol	lactate	formate	H_2 gas	no supplement
3-CP	56.98	46.37	52.39	42.37	62.53
4-CP [1)]	57.68	68.59	56.96	81.30	70.91
2,4-DCP [2)]	68.22	78.70	72.23	69.76	80.44
2,5-DCP [2)]	61.05	70.28	66.91	74.70	65.42
2,6-DCP [2)]	38.71	35.71	33.97	56.27	31.71
3,4-DCP	32.62	44.13	39.94	43.69	39.50
2,3,6-TCP	47.78 [3)]	60.04 [3)]	53.01 [3)]	33.57 [3)]	60.40 [3)]
2,4,6-TCP	56.33 [4)]	64.29 [4)]	54.80 [4)]	49.16 [1)]	57.59 [4)]
2,4,5-TCP	77.89	72.95	71.51	64.12	73.67
PCP	60.38	52.31	56.73	45.45	54.18

Note : 1) % transformation after 1 day of incubation

2) initial concentrations are between 1.1-1.3 mM

3) % transformation of 3-CP, a product from reductive dechlorination of 2,3,6-TCP

4) % transformation of 4-CP, a product from reductive dechlorination of 2,4,6-TCP

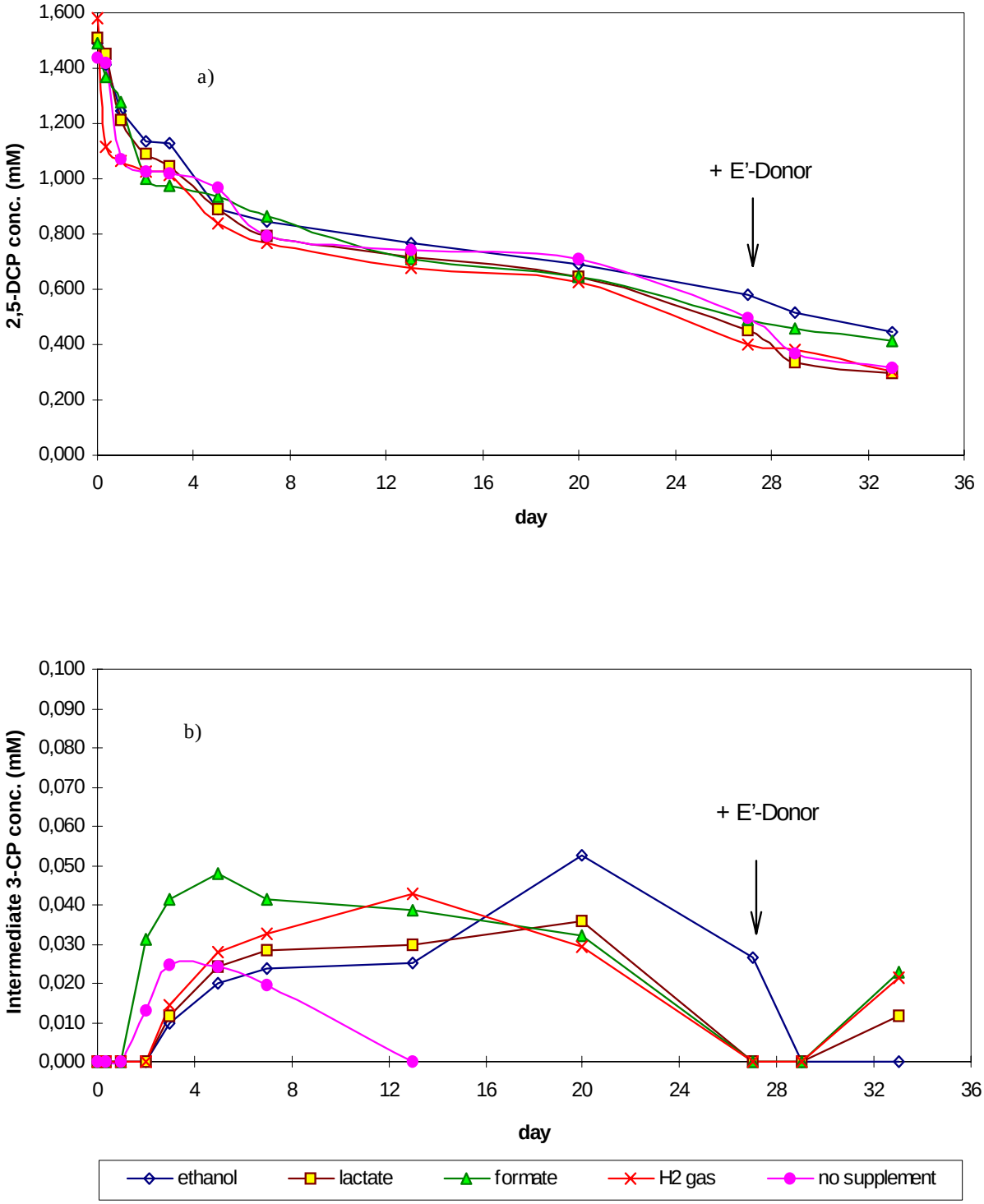


Fig. 3.32 Transformation of 2,5-DCP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 2,5-DCP (b) concentration of 3-CP (intermediate from dechlorination of 2,5-DCP).

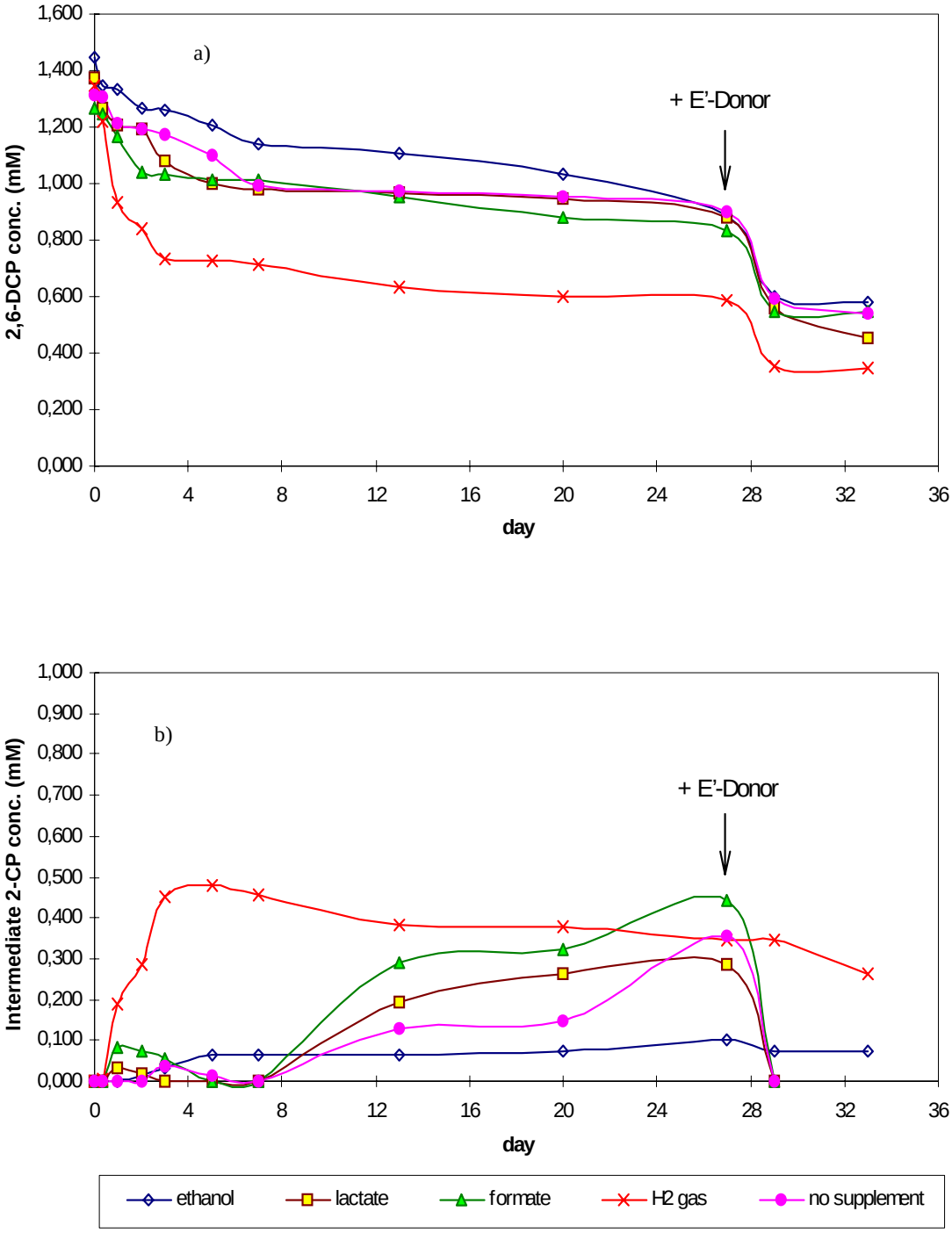


Fig. 3.33 Transformation of 2,6-DCP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 2,6-DCP (b) concentration of 2-CP (intermediate from dechlorination of 2,6-DCP).

Either in the presence or absence of an electron donor, 2,4,5-TCP was sequentially dechlorinated via 3,4-DCP to 3-CP (Fig. 3.34 a-c) and most of it was transformed when incubated with lactate or H_2 gas (Table

3.3). The accumulation of 3,4-DCP in the samples which had ethanol as electron donor and accumulation of 3-CP in samples which were dosed with lactate and formate was observed during the incubation (Fig. 3.34 b, c). Transformation of other TCPs was a little different from that of 2,4,5-TCP. In the samples which were incubated with H_2 gas, 2,3,6-TCP was sequentially dechlorinated to 2,5-DCP and 3-CP (Fig 3.35 a) whereas 2,3,6-TCP in other samples was immediately double dechlorinated to 3-CP, which was further transformed by the cultures (Fig. 3.36). For the removal of 3-CP, non-amended samples and samples incubated with lactate showed the highest transformation efficiency (Table 3.3). The cultures required approx. 27 days to remove 2,4,6-TCP from the samples when H_2 gas was used as an electron donor (Fig 3.35 b). Besides 4-CP, 2,4-DCP was found as one of the intermediates of 2,4,6-TCP transformation by 2-CP degrading cultures in samples which were incubated with ethanol and lactate (Fig. 3.37). 2,4,6-TCP in other samples was rapidly dechlorinated to 4-CP, which was completely removed within 3 days

Most of the PCP was removed at the begining of the incubation like the transformation of PCP in samples amended with glucose (Fig. 3.38 a). Among four electron donors, ethanol showed the greatest efficiency on PCP transformation (Table 3.3). Many intermediates detected by HPLC were found from the transformation of PCP. Based on the retention time, two interesting intermediates (designed intermediate 3 and intermediate 4) were assumed to be one of the less-chlorinated phenolic compounds although none of their retention times matched with those of available reference compounds. Intermediate 4 was suspected to be tetrachlorophenol (TeCP) because its retention time was between TCP and PCP. Intermediate 3, whose retention time was between phenol and 2-CP, might have been catechol, one of the products from hydrolytic dechlorination.

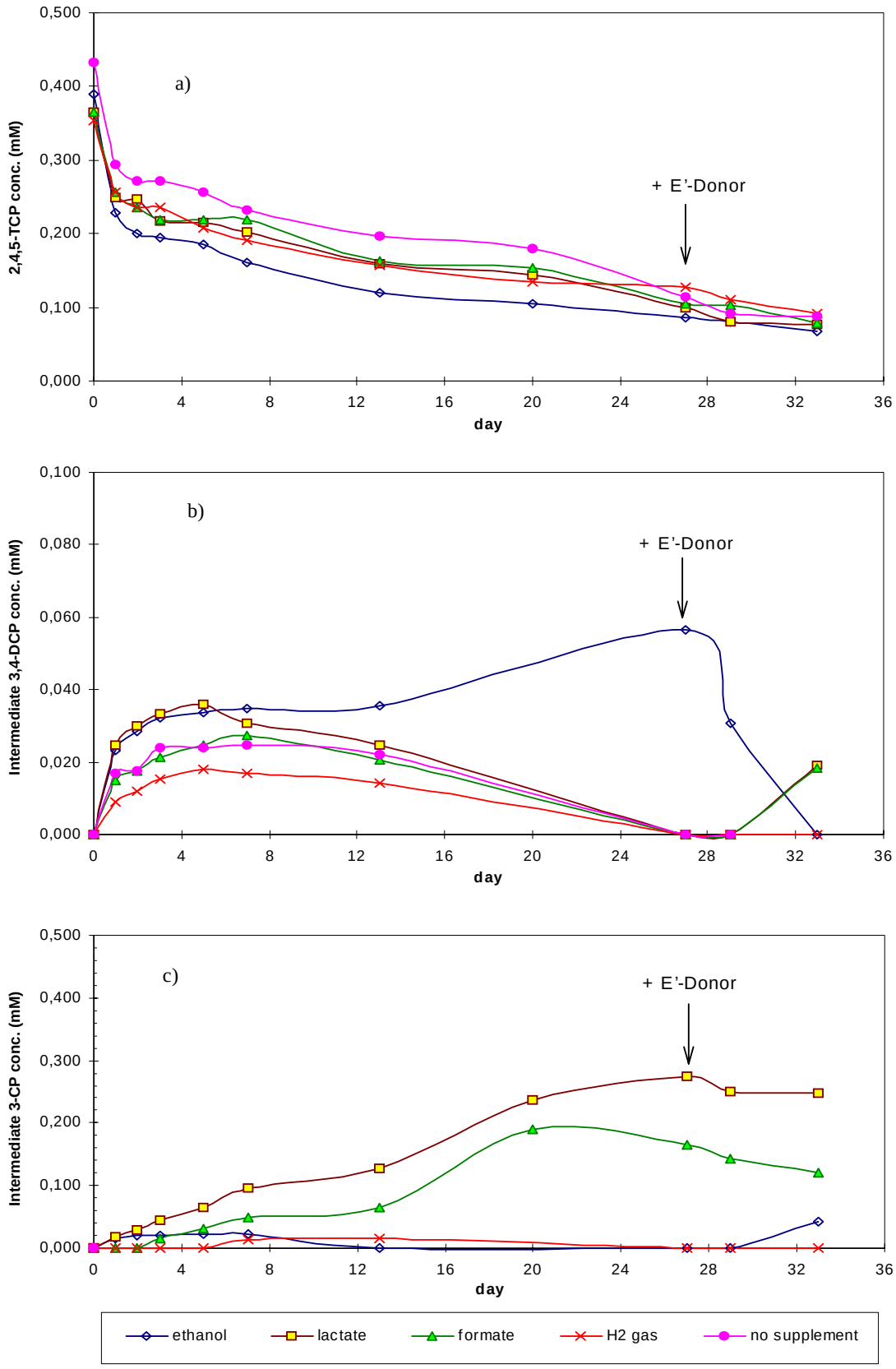
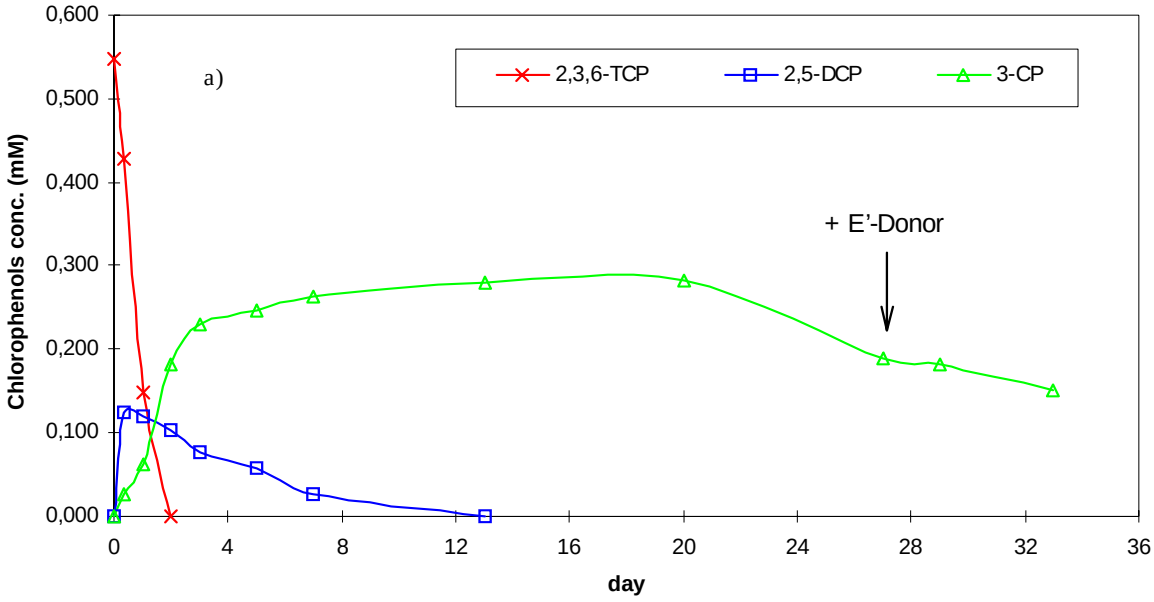


Fig. 3.34 Transformation of 2,4,5-TCP by 2-CP degrading cultures amended with various electron donors. (a) concentration of 2,4,5-TCP (b, c) concentration of 3,4-DCP and 3-CP (intermediates from dechlorination of 2,4,5-TCP).



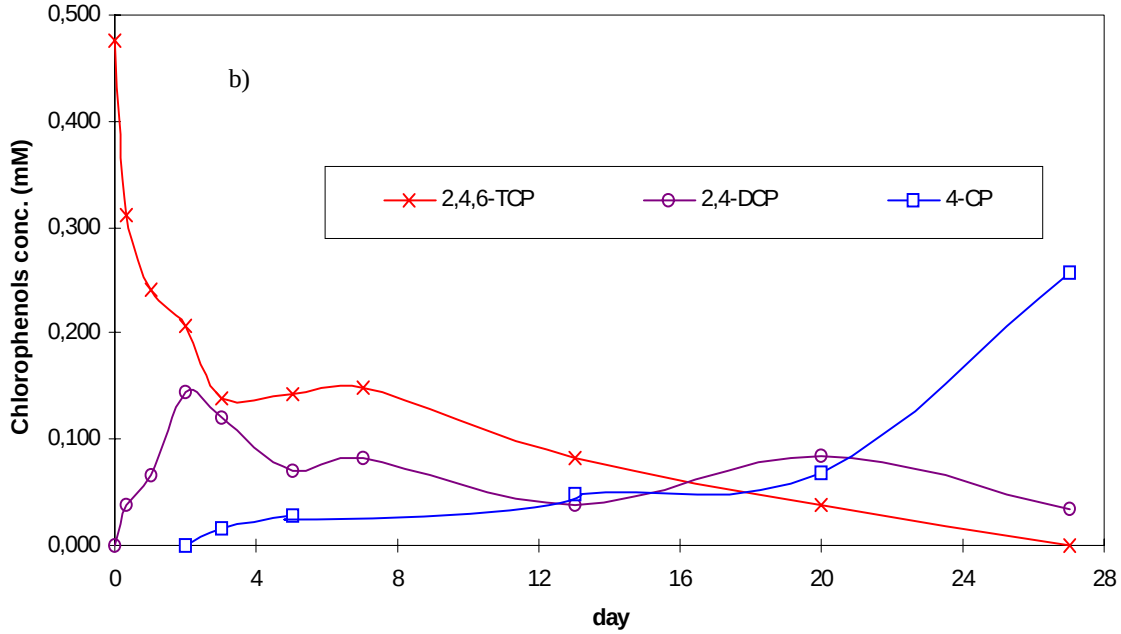


Fig. 3.35 Transformation of 2,3,6-TCP (a) and 2,4,6-TCP (b) by 2-CP degrading cultures amended with H_2 gas as electron donor.

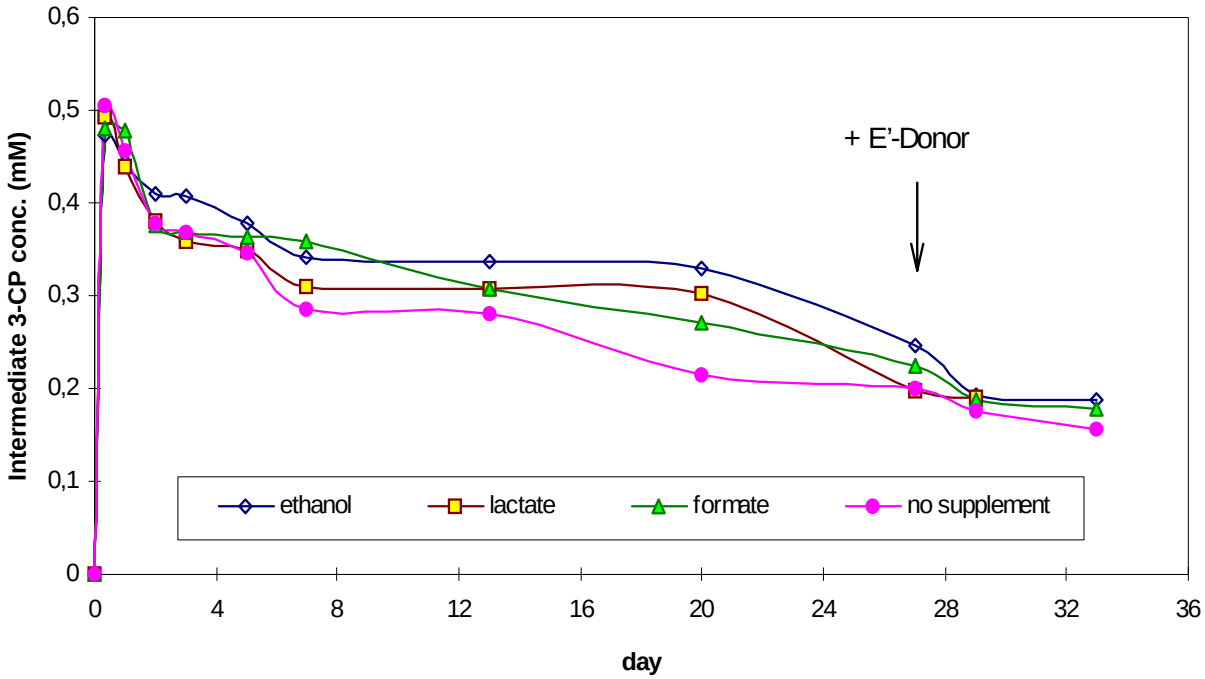


Fig. 3.36 Concentration of 3-CP (intermediate from the dechlorination of 2,3,6-TCP) in 2-CP degrading cultures amended with various electron donors.

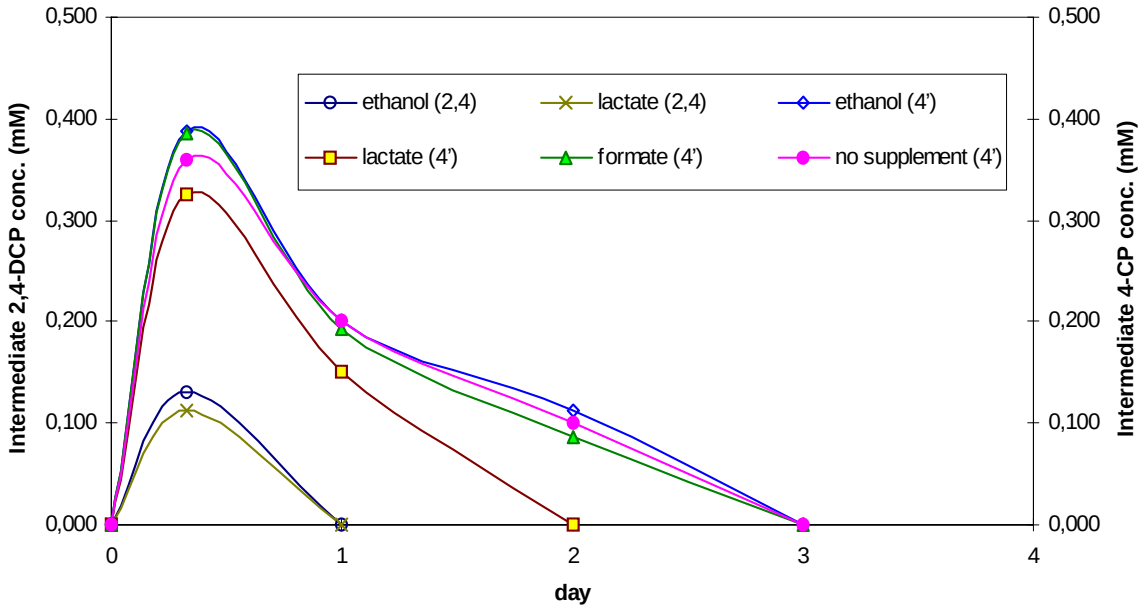


Fig. 3.37 Concentration of 2,4-DCP and 4-CP (intermediates from the dechlorination of 2,4,6-TCP) in 2-CP degrading cultures amended with various electron donors.

The amount of these two intermediates, in terms of peak area, in each sample is shown in Figure 3.38 (b, c). It should be noted that phenol was found as the intermediate in samples incubated with H_2 gas instead of

intermediate 3 while in other samples no phenol was detected. The highest amount of intermediate 4 was found in samples amended with ethanol and the highest amount of intermediate 3 was observed in non-amended samples.

On day 27 of the experiment the same amount of each of the electron donors as initially was added to samples in which the transformation was not complete. In non-amended cultures, 5 mM of glucose was used as electron donor. The decline of each chlorophenol and of their intermediates was obviously seen in almost every sample after a few days of incubation (Fig. 3.29-3.38). These results confirmed the stimulatory effect of electron donors on the dechlorination of chlorophenols.

3.10 Biosorption of PCP by anaerobic granular sludge

In the evaluation of the potential of 2-CP degrading granules for degradation of PCP in the presence of various electron donors (see 3.9.1 and 3.9.2), it was observed that the amount of intermediates evtl. chloride ions did not correspond with the quantity of PCP that disappeared. According to a number of studies PCP adsorbed to anaerobic granular sludge (Kennedy et al., 1992; Kennedy and Pham, 1995). It was assumed that some PCP was adsorbed by the 2-CP degrading granules. To confirm this hypothesis, the biosorption of PCP to 2-CP degrading granules at different concentrations was investigated using batch serum bottle tests.

Figure 3.39 illustrates the concentration of PCP that was found in supernatant of control and tested samples at each time interval (initial PCP conc. was 40 mg/l). Approximately 30 % of PCP that was added into the samples was immediately adsorbed to the biomass and/or glass bottles. The difference between PCP concentration in supernatant of the

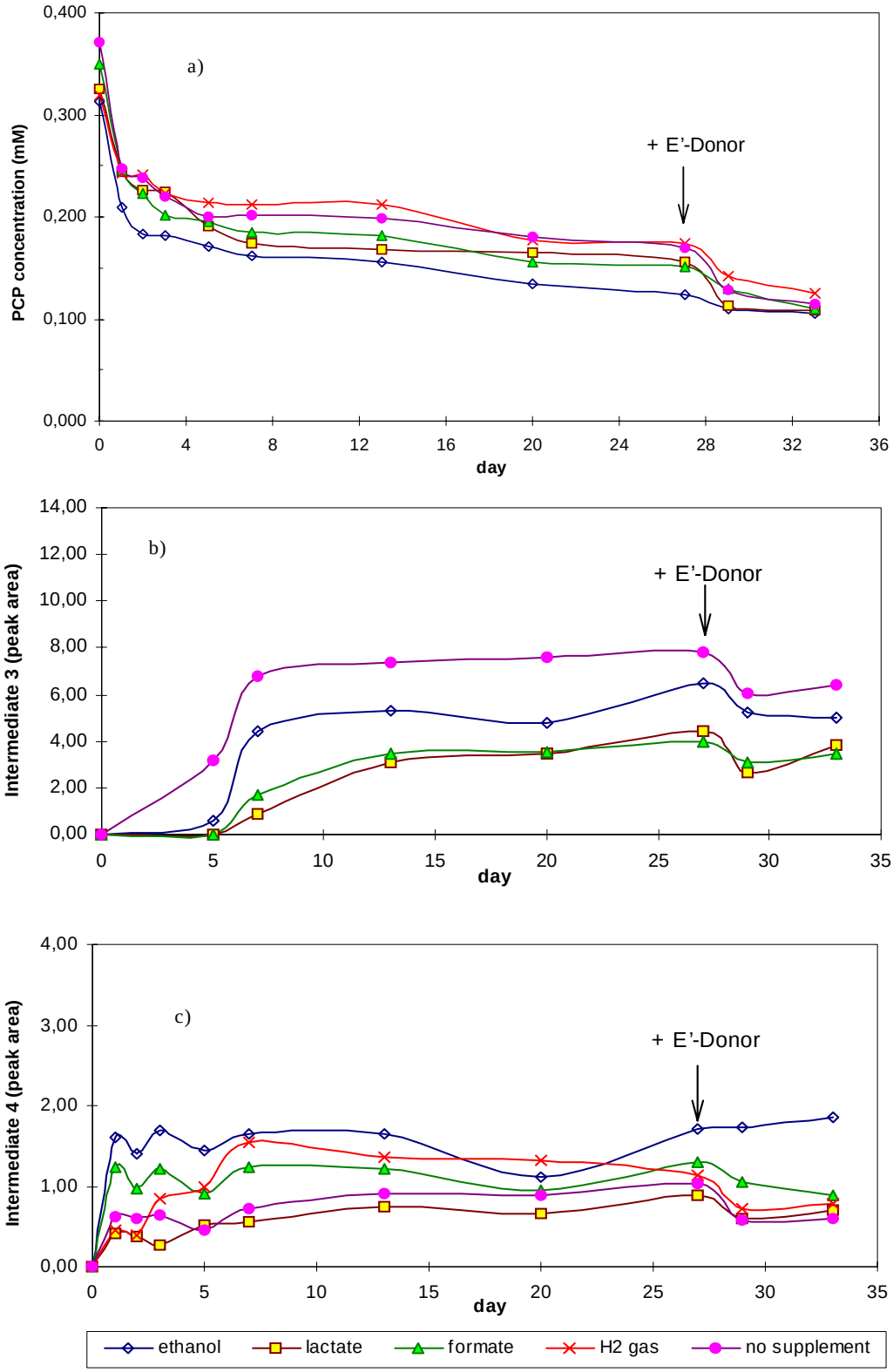


Fig. 3.38 Transformation of PCP by 2-CP degrading cultures amended with various electron donors. (a) Concentration of PCP; (b, c). Amount of intermediate 3 and intermediate 4, expressed in form of peak area, occured during the transformation of PCP by 2-CP degrading cultures (Intermediate 3 might be catechol, intermediate 4 might be tetrachlorophenol).

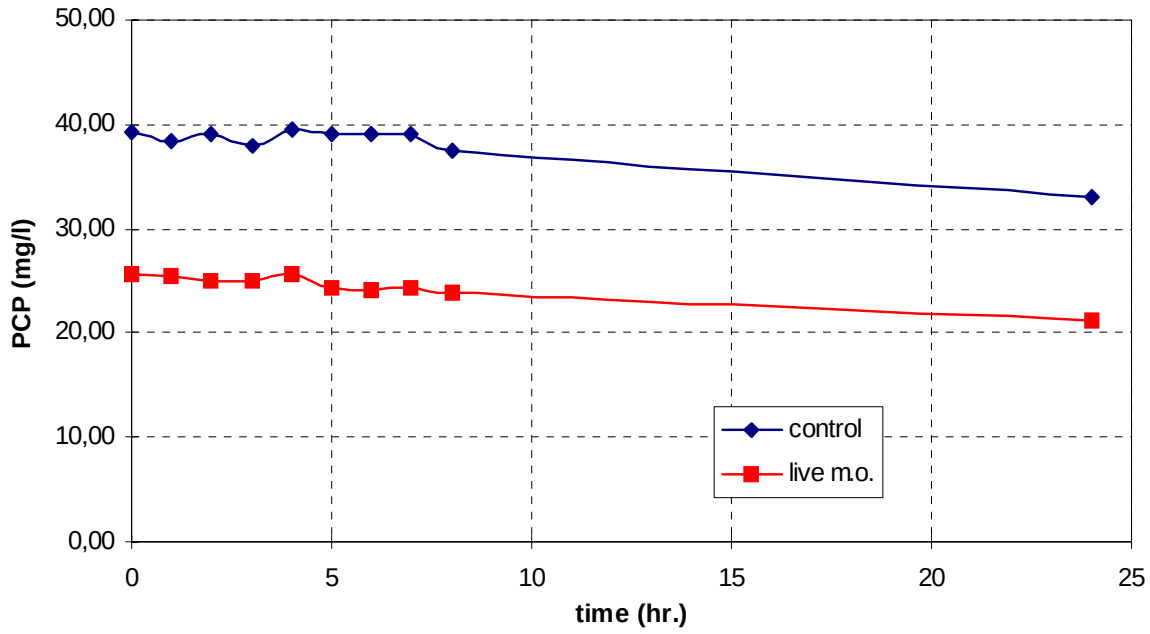


Fig. 3.39 Concentration of PCP in supernatant of control and tested biomass at each time interval.

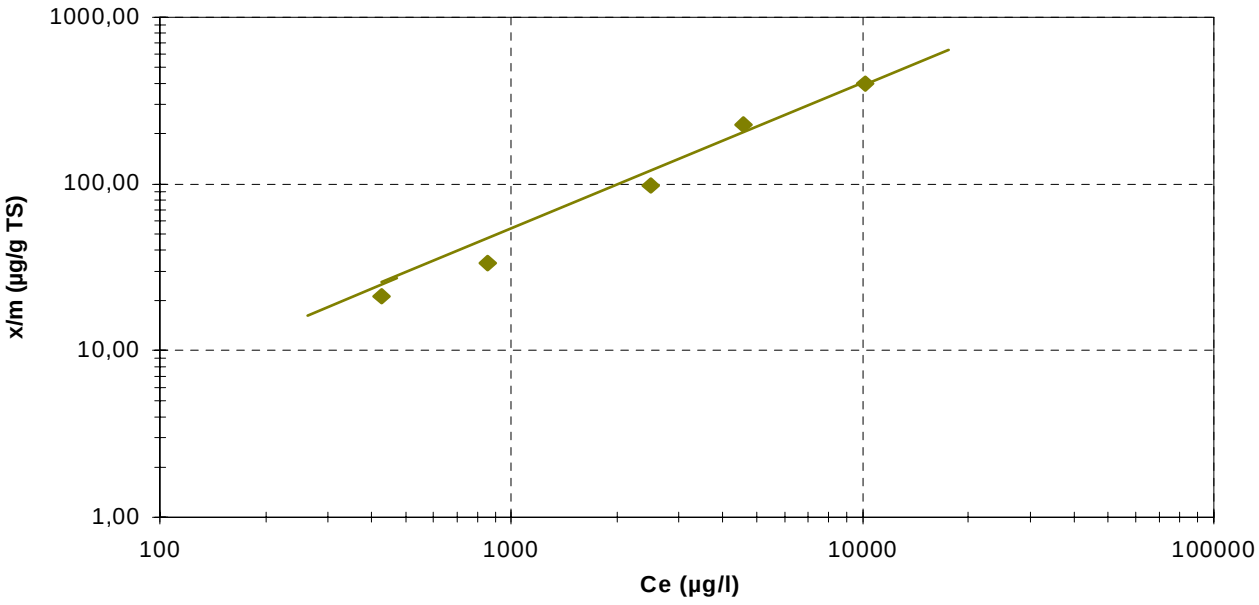


Fig. 3.40 Biosorption of PCP by living anaerobic granular biomass at 35°C.

control and the tested samples was relatively constant during 24 hours of incubation. The concentration of PCP used for determination of the biosorption isotherm of PCP was between 0.5-20 mg/l. Equilibrium conditions were determined after 3 hours of contact time to avoid the transformation of PCP by the tested granules although the results from the above test showed that the concentration of PCP in tested samples was constant over 24 hours of contact time. Figure 3.40 shows live anaerobic granular biomass biosorption data by the Freudlich equation isotherm. Non-linear least squares analysis were performed on the equilibrium data to determine the Freundlich constant, which gave K (Freundlich constant) = 5.48 x 10^{-2} and $1/n$ (exponent) = 0.97. The isotherm fitted the Freundlich equation very well, with a correlation coefficient greater than 0.9.

4 DISCUSSION

4.1 Performance of a UASB reactor for the treatment of glucose-containing synthetic wastewater (reactor R 1)

Fang and Chui (1993) demonstrated that the COD removal efficiency of a UASB reactor was mainly dependent on the COD loading rate, which could be adjusted by varying the HRT and/or the COD level in the wastewater. Typical HRTs for UASB reactors range from 8 to 36 hours (Kennedy et al., 1992). A HRT of 8 h was selected for operating reactor R 1 in order to obtain high granules production for use in the other experiments.

The system worked well at the set loading rate but could not tolerate a temperature shock. After the temperature shock a low biodegradation activity remained, which had a high sensitivity to changes of the organic loading and a change in the type of granules (clumped granules) was observed. Various attempts to restore the system to its original activity failed. Heating of granules for a short time did not completely destroy the activities of the granules and some biogas production could still be observed. The flotation of granules after the temperature shock may be not only due to high gas adsorption during and after heating. Blaszczyk et al. (1994) observed hollow cores, which were found in the granules that float out of the reactor when effluent from a Corn Wet Milling corporation (Casco, London) was treated. It has been postulated that the lysis of biomass during shock loading was the main reason for this hollow core formation. Such hollow cores might also be formed within the floating glucose-degrading granules during heating and might cause gas entrappment.

Normally, ammonium nitrogen is utilized for biomass production in anaerobic systems. Different nitrogen demands of anaerobic processes, ranging from a C:N ratio of 50:1 to 16:1, have been reported in the literature (cited in Thiel, 1990). The C:N ratio of 18:1 used in the described experiments was apparently not exactly the appropriate proportion since ammonia remained unused in the effluent. Low nitrogen removal may be due to the fact that biomass grew in granules (as a form of immobilization) at short HRT (8 h). The nitrogen elimination increased a little when the reactor was operated at a HRT of 24 h. In reactors with suspended biomass, more biomass formation for the same degradation activity is required and a C:N ratio of 18:1 seems to be ideal.

4.2 Acclimation and enrichment of MCP-degrading granules

The chemical structure and the concentration of halogenated substrates were important factors influencing the length of the acclimation period and the ultimate degradation (Linkfield et al., 1989). Furthermore, the hydrophobicity of many haloaromatic compounds prevented or reduced their availability to microorganisms and thus affected their biological dehalogenation and degradation. Therefore, it was important to determine the degradation potential (at low concentration) and inhibition levels of each MCP (at higher concentration) of unacclimated sludge for successful establishment of MCP-degrading granules. In addition, other factors that affect the aquisition of biological dehalogenation by these non-adapted cultures, e.g. electron donors and pH, should also be evaluated to provide optimal conditions under which the most extensive reductive dechlorination would occur.

The preferential removal of chlorine from the *ortho* position in the neighborhood to the hydroxyl group by unacclimated sludges, observed in this study, was consistent with results that have been reported for acclimated and unacclimated microbial consortia by many researchers (Boyd and Shelton, 1984; Magar et al., 1995; Mikesell and Boyd, 1986; Nicholson et al. 1992; Wood et al., 1989). Apart from the position of the chlorine substituent, the initial concentration of MCP seemed to have also an effect on the transformation (see Table 3.1). Results from the toxicity assay showed that the methane production of unacclimated cultures was only slightly inhibited (≤ 40 %) when the MCP concentration was ≤ 2 mM. The toxic effect increased in relation to the position of the chlorine substituent on the benzene ring from : 3-CP > 4-CP > 2-CP (see Fig. 3.5 and 3.7). The results of both experiments were used to arrange the appropriate conditions for acclimatization glucose-degrading granules to each monochlorophenol.

The stimulatory effect of biodegradable organic substrates such as glucose, acetate, propionate and butyrate on the dechlorination of MCPs was observed only in samples which were incubated with 2-CP (Fig. 3.8). By following the decrease of each of the above mentioned organic substances in serum bottles by gas chromatography, it turned out that the disappearance of these substrates in samples which were incubated together with 3-CP and 4-CP was much slower than in the samples that contained 2-CP (data not shown). The degradation of those organic substrates was assumed to be retarded by the poisonous effect of 3-CP and 4-CP. This might be the explanation why these organic substrates could not stimulate the dechlorination of 3-CP and 4-CP. Kuhn et al. (1990) hypothesized that the stimulation effect of organic substrates on dehalogenation reactions was due to a transient increase in H_2 concentration in the medium and that H_2 was an electron donor for dehalogenation. Indeed, it was not surprising that glucose had the greatest stimulatory effect on dechlorination of 2-CP (see Fig. 3.8) since acetate, propionate and butyrate are common products of glucose degradation besides some hydrogen. Considering the 4 tested organic substrates as the source of reducing equivalents required for dechlorination of 2-CP, the numbers of H-atoms in their molecules corresponded with the dechlorination rate which increased in the order from glucose > butyrate > propionate > acetate. Alternatively, these organic substrates might directly support the growth of dehalogenating organisms or support the growth of non-dehalogenating organisms to provide optimal conditions for syntrophy or a population succession that stimulates the dehalogenation activity (Mohn and Teidje, 1992).

The physical factors such as pH of the medium may also play an important role in dechlorination of chlorophenols. In their studies on the effect of pH on the dehalogenation of 2,4,6-TCP, Armenante et al. (1993) found that dechlorination occured only when the pH was within the range of 8.0-8.8. The dechlorination of 2-CP in this study was found to occur when the pH was in a wider range, between 7.0-8.5 (see 3.3.2 and Fig 3.10). The slight difference in optimal pH range of both chlorophenols was supposed to be due to the number of the chlorine substituent.

4.3 Performance of UASB reactor for the treatment 2-CP containing synthetic wastewater (reactor R 2)

A high efficiency of 2-CP removal was achieved a few day after start-up. This may be the result of operating the reactor at optimal conditions, which were elaborated in previous experiments. In addition, recirculation of part of the effluent from the reactor together with the incoming wastewater helped to lower the

initial concentration of 2-CP in the influent. By this procedure, the granules that possessed the 2-CP-degrading capability could be successfully enriched in the UASB reactor.

The attempt to reduce the HRT from 4.5 day to 2.8 days at no change of 2-CP loading rate led to a hydraulic loading shock (see 3.4.1). The shorter HRT seemed to have a more pronounced negative effect on the 2-CP degradation than on COD degradation. As can be seen in Figure 3.11 b and Figure 3.11 c the 2-CP removal efficiency dropped immediately from 97 % to 75 % while the COD removal efficiency was only 12 % decreased (from 90 % to 78 %). Based on the experimental results from start-up until day 90 (before loading shock) approx. 130 mg of 2-CP in the influent was mineralized per day when the reactor was operated at a HRT of 4.5-5.0 day. The decreasing in the 2-CP removal efficiency at the same 2-CP loading rate of 50 mg/l, d due to the shorter HRT indicated that the dehalogenating bacteria in the reactor required more time than 2.8 days to mineralize the above mentioned amount of 2-CP.

System disruption caused by a temperature change on day 179 not only interrupted the recovery of the 2-CP removal efficiency from the previous loading shock but also deteriorated COD, 2-CP and phenol degradation of the system. However, 2-CP degrading organisms seemed to tolerate the high temperature better than phenol-degrading organisms and the organisms which degraded glucose. This can be seen from the drastic decreasing of COD removal and the appearance of phenol, that has not been detected before in the effluent, while the 2-CP dehalogenation efficiency was reduced only 20 % (see Fig 3.11 a, b, c). The lower sensitivity to sudden changes in the anaerobic system (loading, temperature, O_2) of dehalogenating organisms in comparison to phenol-degrading organisms was also observed by Dietrich (1990). Although the damage from the changes of HRT and temperature was not total, the recovery was gradual and lengthy (approx. 30-50 days for each case).

When the full recovery of bioacitvity was attained, the development of 2-CP degrading granules was further improved. A 2-CP degradation rate up to 0.6 g/l, d with a removal efficiency of ≥ 90 % at the end of the experiment (see Fig.3.11 b) was higher than the rates reported elsewhere. Dietrich and Winter (1990) reported a 2-CP degradation rate of 0.18 g/l, day in suspended cultures and 0.375 g/l, d in cultures immobilized on Liapor clay beads. Even in anaerobic fluidized-bed granular activated carbon (GAC) treating wastewater containing inhibitory concentrations of different chlorophenols, a 2-CP degradation rate of only 0.164 g/l, d was obtained (Flora et al., 1994).

While net chloride production proved dechlorination of 2-CP in the reactor, the mineralization of 2-CP to CH_4 and CO_2 was indicated by the increasing of biogas production along with the increasing 2-CP loading rate. Based on the fact that a constant glucose concentration (0.9 g/l) was used throughout the experiment, a

corresponding amount of biogas to the amount of glucose in the influent should be obtained if mineralization of 2-CP did not occur. Estimation of the biogas quantity that could be produced from glucose degradation during the last phase of the experiment (day 450-550) resulted in a total biogas quantity of 1075-1120 ml/d. In the same period of the experiment, a biogas production rate of between 1400 and 1700 ml/d was observed, indicating that biogas in the reactor was not only derived from glucose degradation (see Fig. 3.11 d). Since no intermediates were found in the effluent, the reduced biogas production between day 470-530 and at the termination of the experiment was supposed to be due to the toxic effect of 2-CP at high concentration on methanogens, which then affected the quantity and quality of the biogas (proportion of CH_4 in biogas reduced from 60 % to 50 %).

4.4. Mineralization and dechlorination kinetics of 2-CP in batch cultures

Reductive dehalogenation involved the removal of a halogen substituent from a molecule with concurrent addition of electrons (Mohn and Tiedje, 1992). As shown in Fig. 3.8, Fig. 3.13, Fig. 3.19 and Fig. 3.30-3.38, stimulation of reductive dechlorination or enhancement of that activity was achieved when organic substrates or an electron donor were added to the cultures. However, dechlorination also occured eventhough such substrates were absent. This phenomenon might be explained by some nutrients or components of the medium or by the intermediates of phenol degradation that probably served as electron donors or supported the growth of the dehalogenating organisms.

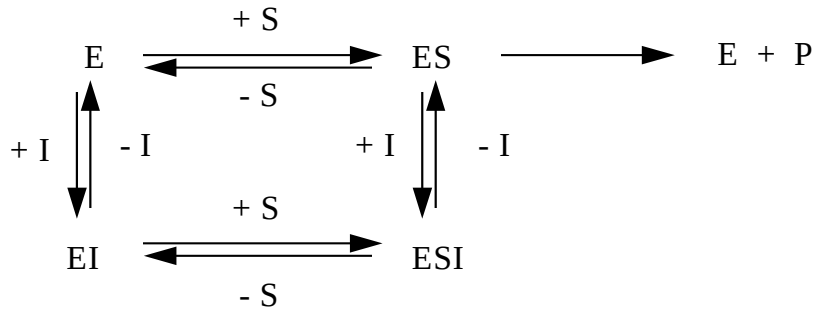
Mineralization of MCP by anaerobic cultures, both in batch and continuous cultures, has been reported by many researchers (Boyd and Shelton, 1984; Dietrich and Winter, 1990; Häggblom and Young, 1995; Krumme and Boyd, 1988; Sharak Genthner et al., 1989). In this study, 2-CP-degrading granules were developed in an UASB reactor by using glucose-

degrading granules that were acclimated to 2-CP. This biomass had shown the capability of mineralization of 2-CP to methane and carbon dioxide in an UASB reactor (see Fig. 3.11 a, c) and in batch cultures in the presence / absence of glucose as co-substrate (see Fig. 3.13-3.14). The results indicated that the mineralization rate of these cultures might have been limited by the phenol degradation at high 2-CP concentration, but phenol was rapidly removed when no 2-CP was in the samples. Moreover, the dechlorination rate could be enhanced by subsequent refeeding of the respective chlorophenols and supplementary substrates.

By continously increasing the 2-CP load in the influent, a high dechlorination rate in the UASB reactor was obtained (compare Fig 3.13 b and Fig. 3.15-3.16). The K_m value of 152 mg/l (1.18 mM) showed that this dechlorinating cultures had a high affinity for 2-CP. This indicated that the degradation of 2-CP by this cultures was a first-order reaction, in which dechlorination/degradation rates directly depended on concentration of 2-CP. This dependence was no more observed when granular sludges taken from reactor R 2 on day 462 was used for the specific dechlorination rate determination. It is unlikely to suppose that the cultures had reached their maximum dechlorination rate and then exhibited zero-order kinetics because the specific dechlorination rates of some 2-CP concentrations were lower than the rates obtained before (0.07-0.08 mM/h opposed to 0.06-0.18 mM/h). Results suggest that inhibition of 2-CP dechlorination had occured at high concentration of 2-CP. The inhibition of 2-CP dechlorination at high concentration can be seen in Figure 3.19 c, where approx. 2 mM of 2-CP could be dechlorinated from an initial concentration of 5 mM whereas this culture was able to completely dechlorinate 3 and 4 mM of 2-CP (Fig. 3.19 a, b). This may be attributed to the toxicity of high 2-CP concentrations on the granules since the 2-CP concentration in the reactor and in the influent at that time (day 462 of reactor operation) was $\leq$ 5 mM (see Fig. 3.11 a). In addition, the degradation of glucose was also observed to be delayed in samples which were incubated with 5 mM of 2-CP.

4.5 Kinetics of glucose degradation

The inhibition of glucose degradation by 2-CP which was found in the samples incubated with 5 mM of 2-CP was seen in several experiments (see Fig. 3.20). For determination of kinetic parameters for glucose degradation by adapted and non-adapted biomass, the method of Cornish-Bowden (1974) was used together with the method of Dixon (1953), because the later itself was not sufficient to distinguish between the four types of simple linear inhibition (i. e. competitive, uncompetitive, non-competitive and mixed inhibition). By using both methods, it can be concluded that 2-CP caused a mixed inhibition of glucose degradation by adapted and non-adapted biomass. Mixed inhibition can actually arise from several situations but in this experiment the simplest one was assumed to occur, in which EI had a lower affinity than E for S and the ESI complex was nonproductive. The equilibria describing this system are shown below.



dissociation constant of EI complex, $Ki = \frac{[E][I]}{[EI]}$

dissociation constant of ESI complex, $Kii = \frac{[ES][I]}{[ESI]}$

As long as I is present, some of the enzyme will always be in the nonproductive ESI form, even at an infinitely high [S]. Consequently , $V_{maxi} < V_{max}$. At any [I], a portion of enzyme available for reaction with S will exist in the lower affinity EI form, consequently, $K_{mapp} > K_m$.

The high K_m values of adapted biomass (approx. 15 times more than that of non-adapted biomass) indicated that the degradation of glucose by this biomass was strongly dependent on glucose concentration while for non-adapted biomass it was less dependent, although V_{max} of non-adapted biomass was lower than that of adapted biomass. This may be explained by the higher capability to degrade glucose of non-adapted biomass compared to adapted biomass, which was aimed to degrade 2-CP more than glucose. Moreover, when considering the overall inhibition constant values (K_i and K_{ii}) of both biomasses, the inhibition of glucose degradation in the adapted biomass system tended to change to the uncompetitive type (i. e. I binds only with ES complex).

4.6 Degradation potential of 2-CP-degrading granules for other chlorophenols

4.6.1 MCP, DCP and TCP

In the evaluation of the degradation potential of 2-CP degrading granules for other chlorophenols, the chlorine substituents in *ortho* position to the hydroxyl group of 2,4-DCP, 2,5-DCP and 2,4,5-TCP were first removed as expected (see Fig. 3.25 a, c and Fig. 3.26 b). For chlorophenols containing two ortho chlorines (2,6-DCP, 2,3,6-TCP, 2,4,6-TCP), this cultures could simultanously dechlorinate both chlorine atoms (see Fig. 3.25 d

and Fig. 3.26 a, c). Besides the capability in dechlorinating chlorine substituents from the *ortho* position, this culture could also remove chlorine substituents at *meta* and *para* position (see Fig. 3.24, Fig. 3.25 a-c, Fig. 3.26 a-c). However, this ability was limited for chlorophenols that had chlorine substituents at both positions (3,4-DCP and 2,4,5-TCP). This limitation was supposed to be due to the position of chlorine substituents that are next to each other which makes them difficult to attack or one chlorine substituent has a negative effect on the removal of the other. An effect of one substituent on the dehalogenation of substituents at other positions of chlorophenols was observed in the study of specificity of reductive dehalogenation by *Desulfitobacterium dehalogenans* JW/IU-DC1 (Utkin et al., 1995). They found that chlorine substituents at carbon 5 (second meta position) had a negative effect on the dehalogenation rate or prevented dechlorination at the position 2. Additionally, it can be seen in Fig. 3.24 that the cultures required several days to remove one chlorine substituents at *meta* or *para* position. Thus, it should be more difficult for these cultures to dechlorinate chlorophenols containing two *meta* or two *para* chlorine substituents or have chlorine atom at both *meta* and *para* position. The appearance of both 3-CP and 4-CP as intermediates in the transformation of 3,4-DCP and 2,4,5-TCP (see Fig. 3.25 b and Fig. 3.26 b) indicated the removal of chlorine atoms at both positions but with a rate that was slower than dechlorination of chlorophenols containing only one *meta* or *para* chlorine substituent.

The capability in dechlorinating chlorine substituent at *meta* and *para* position by these cultures may be explained by the fact that the population which was capable of removal *meta* or *para* chlorine substituents still existed in the consortium. Another explanation would be that this capability may be a result of cross-acclimation between 2- and 4- chlorophenol which resembled the *ortho-para* cross-acclimation pattern observed in acclimated sewage sludge (Boyd and Shelton, 1984). The removal of a chlorine at the *meta* position by this culture was surprising since *ortho-meta* cross-acclimation patterns have so far not been reported anywhere. A possible explanation would be that this cultures might consist of a unique population with a somewhat broader substrate range. However, this capability (*meta* dechlorination) seemed to diminish when no electron donor or electron donors other than glucose were added to the cultures, especially in samples incubated with 3-CP (Fig. 3.29 a). It was assumed that ethanol, lactate, formate and H_2 gas had negligible or even a negative effect on the dechlorination of chlorine atom at the *meta* position since 3-CP was most transformed in non-amended samples. Nontheless, the enhancement of *meta*-Cl substituent removal could be seen in most of the samples to which those electron donors were added after 27 days of incubation (see Fig. 3.29 a, Fig. 3.30 b, Fig 3.32 b, Fig. 3.36). Alternatively, the population which was responsible for *meta*-Cl substituents removal might have been decreased due to the selective pressure exerted to steadily 2-CP-fed granules in reactor R 2 [note that granules

used in testing with glucose and other electron donors were taken from the reactor at different times]. On the contrary, the dechlorination rate of chlorophenols containing *para* Cl substituents was markedly improved (see Fig.3.29 b, Fig. 3.30 a, Fig. 3.34 b and Fig. 3.37). The ability of cross-acclimation between *ortho*- and *para*-Cl substituents of these cultures may have been the reason for the increase of the removal rate of chlorine substituents at the *para* position which was consistent with a high 2-CP dechlorination rate. It should be noted that the increase of the dechlorination rate at the *para* position was not the effect of an electron donor because this increase was also observed in non-amended samples.

The selected granules appeared to have sufficient endogenous reductants to support extensive dechlorination, as samples with no addition of external electron donors could dechlorinate all examined chlorophenols over the incubation period (see Fig. 3.29-3.38). According to the results from experiments with electron donors other than glucose, it is apparently that most of these electron donors added to the samples did not enhance the dechlorination rate or transformation efficiency of this cultures when compared with non-amended samples (see Table 3.3). Some electron donors had positive effect on the transformation of one chlorophenol but negative effect on the others. For example, H_2 gas enhanced the transformation of 4-CP, 2,5-DCP, 2,6-DCP and 3,4-DCP but retarded or inhibited the dechlorination of 2,3,6- TCP and 2,4,6-TCP (see Fig. 3.35 a, b). Nevertheless, there was no relationship between the position of the chlorine substituents and the effect of each examined electron donor on transformation of chloropenols.

The effect of the initial concentration of the parent chlorophenol on rate and extent of dechlorination can be seen in the transformation of 2,4-DCP, 2,5-DCP and 2,6-DCP (Fig. 3.31-3.33). A high initial concentration (approx.2.0-2.5 times higher than those in samples incubated with glucose) decreased the transformation efficiency and inhibited reductive dechlorination of both parent compound and less-chlorinated intermediates. The transformation of 2,6-DCP seemed to be most affected by a high initial concentration compared to the other three DCPs. A similar effect of initial chlorophenol concentration as found in this experiment was also observed by Mohn and Kennedy (1992). In their batch experiments, sequential reductive dechlorination of 2,3,6-TCP via 2,5-DCP to 3-CP by sludge granules from an UASB reactor which received 2,3,6-TCP as substrate in the influent, occured when the 2,3,6-TCP concentration was between 50 µM and 1.75 mM. At higher substrate concentrations, 2,3-DCP appeared as a product and 3-CP was not detected. Madsen and Aamand (1992) also reported a complete dechlorination of 10 and 20 µM 3,4,5-TCP to 3,5-DCP in 2 days, while an initial concentration of 40 µM 3,4,5-TCP seemed to be toxic for the mixed cultures and 3,4,5-TCP was only slowly transformed.

The non-stoichiometric loss of mass in the transformation of 3,4-DCP, 2,4,5-TCP and PCP was suspected to occur by adsorption to the granules. Anaerobic granules are known to be very porous (Macleod et al., 1990; Wiegant and de Man, 1986) allowing penetration and adsorption. The reason for the adsorption of chlorophenols to sludge granules which were incubated with 3,4-DCP, 2,4,5-DCP and PCP may be the persistence of these chlorophenols to be transformed. In their study of biosorption of chlorophenols to anaerobic granular sludge, Kenndy and Mohn (1992) found that PCP was more strongly sorbed than the less-chlorinated phenols and biosorption of chlorophenols having chlorine in the *para* position or containing two *meta* chlorine tended to be higher than chlorophenols containing only *ortho* or single *meta* substituents. Additionally, 2-CP was reported to have a very low biosorption capacity. Their results are in agreement with the sorption phenomenon observed in this study.

4.6.2 Pentachlorophenol (PCP)

The enriched granules had shown the potential of transforming PCP both in the presence and absence of electron donor (see Fig. 3.27 and Fig. 3.38 a) although about 30 % of disappeared PCP was shown to adsorb to the granules (see Fig. 3.39). Comparing the same incubation time (27 days), glucose seemed to be more effective in increasing the extent of PCP transformation than the other electron donors, since many less-chlorinated phenolic intermediates (i.e. TCP, MCP) including phenol were detected in PCP samples incubated with glucose. Based on the appearance of intermediates, these cultures could remove chlorine substituents in *ortho* position to the hydroxyl group more rapidly than in *meta* and *para* position. Thus, removal of *ortho*-Cl substituents of PCP was expected to occur before removal of *meta*- or *para*-Cl substituents. The unknown isomer of intermediate TCP was suspected to be 3,4,5-TCP which was also found in the degradation of PCP by 2-CP acclimated sludge (Mikesell and Boyd, 1986) while the formation of 2,4,5-TCP suggests *ortho*-and *meta*- dechlorination of PCP. *Ortho*-dechlorination was often found in the pathway of PCP degradation by several acclimated cultures which yielded 2,3,4,5-TeCP or 3,4,5-TCP (Mikesell and Boyd, 1986; Nicholson et al., 1992; Susarla et al., 1996). On the other hand, Hendriksen et al. (1992) reported the degradation of PCP in an UASB reactor by glucose-degrading granules with *para*-dechlorination occuring before *ortho*-Cl substituent removal.

When ethanol, lactate, formate and H_2 gas were used as electron donors, no TCP or MCP were found as intermediates of the transformation of PCP. Furthermore, the transformation pattern of PCP in samples with electron donors other than glucose was not significantly different from that of non-amended samples (see Fig.

3.38 a-c). According to the retention time and possible intermediates in the pathway of PCP transformation by anaerobic bacteria (see Fig. 1.7), intermediate 4 was supposed to be tetrachlorophenol, the first product of reductive dechlorination while intermediate 3 was supposed to be catechol, a product from hydrolytic dehalogenation, a process in which the chlorine substituent is replaced by a hydroxyl group that was derived from water (Fetzner and Lingens, 1994). Low transformation efficiency of PCP by this acclimated culture may be due to the effect of an initial high concentration. In the literature, the concentrations of PCP used for studying PCP degradation by several acclimated cultures were in the range from 1 to 40 µM, which was approx. 10-400 times less than the PCP concentration used in this study.

4.7 Biosorption of PCP by anaerobic granular sludge

The biosorption process has been reported to be very rapid and equilibrium was reached in less than 24 h (Bell and Tsezos, 1987; Tsezos and Bell, 1989). As shown in Fig. 3.39, the constant concentration of PCP in the supernatant from 1 to 8 h after addition indicated that rapid biosorption of PCP to sludge granules occured and equilibrium was reached in less than 2 hours. In order to be sure that biosorption equilibrium had already occured and to avoid transformation of PCP by the granules, a contact time of 2 h was used. Results from HPLC analysis confirmed that during the course of the biosorption test no dechlorinated intermediates were formed. The experimental results fitted well to a Freundlich equation (see biosorption isotherm Fig. 3.40) with the parameters, $K = 5.48 \times 10^{-2}$ and $1/n = 0.97$. The PCP biosorption isotherm determined in this study was compared with those of Kennedy et al. (1992), Kennedy and Pham (1995) and Tsezos and Bell (1989) (Table 4.1).

As seen in Table 4.1, the biosorption capacity for aerobes was higher than for anaerobes. A comparison of the PCP biosorption capacity for granular sludge revealed that the biosorption capacity of granular sludge used in this study was much less than that reported by

Table 4.1 Freundlich parameters and conditions for PCP sorption isotherms by live biomass

	Temp. (°C)	*K*	1/*n*	Reference
Rhizopus arrhizus	20	32.1	0.56	Tsezos and Bell (1989)
Activated sludge	20	85.1	0.60	Tsezos and Bell (1989)
Anaerobic granular sludge (different sludge source)				
lab-scale UASB reactor	35	2.47	0.68	Kennedy et al. (1992)

- Sucrose and acetic acid waste water - 2-CP containing synthetic wastewater	35	5.5×10^{-2}	0.97	This study
full-scale UASB reactor - Pigment	22 ± 1	5.18	0.63	
- Wine	22 ± 1	22.21	0.50	Kennedy and Pham (1995)
- Plant	22 ± 1	2.88	0.72	

Kennedy et al. (1992) and Kennedy and Pham (1995). For an equilibrium liquid phase PCP concentration of 1000 µg/l, biosorption onto 2-CP degrading granules (45.5 µg/g TS) is approx. 6-fold less than for granular sludge developed in laboratory UASB reactor (270 µg/g VSS; Kennedy et al., 1992) and about 9- to 16-fold less than for industrial granular sludge (405-709 µg/g VSS; Kennedy and Pham, 1995). The difference in the biosorption capacity may be related to the source of anaerobic sludge (Kennedy and Pham, 1995). Granular sludge used in this study was developed in laboratory UASB reactor treating 2-CP containing synthetic wastewater, which is different from the others. Furthermore, the 1/*n* value of close to 1 observed in this study, was similar to that of many chlorophenols reported by Kennedy et al. (1992). This value indicated a linear sorption isotherm and suggested a constant-partitioning sorption mechanism. This type of sorption isotherm can occur when the sorbate can readily penetrate into the microbial granule. A linear sorption isotherm commonly occurs when relatively pure, porous sorbents are used and the sorption is being carried out over a relatively small concentration range. It could be argued that some of these conditions were valid in this study because the PCP concentration range was narrow (1 order of magnitude).

5 SUMMARY

Chlorophenols are a class of harzardous chemicals used in industry, as biocides as well as wood preservatives. These compounds are of serious environmental concern because of their widespread usage, toxicity and occurence throughout the environment. Natural removal of chlorophenols from the environment can be achieved by photodecomposition and biodegradation. Anaerobic processes are reported to be suitable for the dechlorination of low to higher-chlorinated phenols while aerobic systems have a tendency to be more suitable for biodegrading the less-chlorinated phenolic compounds.

In this study 2-CP-degrading granules were developed by acclimating bacterial granules which were previously grown on glucose-containing synthetic wastewater to 2-chlorophenol (2-CP). The granules that possessed the 2-CP-degrading capability were then further enriched in a laboratory-scale upflow anaerobic sludge blanket (UASB) reactor at 35°C with 2-CP in the presence of 0.9 g/l of glucose as a co-substrate to supply the required maintenance energy. The physiological and biological factors that affected the biological dehalogenation of those acclimated cultures (e.g. initial concentration of 2-CP, pH of the medium, effect of biodegradable organic substrates) were examined. At the end of the experiment, the granular cultures in reactor were able to treat synthetic wastewater containing 1.03 g (8 mM) of 2-CP/l at a volumetric loading rate of up to 0.6 g/l of reactor volume per day, with a hydraulic retention time of 1.6 day. A 2-CP removal efficiency of more than 90 % was achieved. The enriched biomass showed the capability of mineralization 2-CP to methane and carbon dioxide both in an UASB reactor and in batch cultures in the presence / absence of glucose as a co-substrate. Specific 2-CP dechlorination rates up to 66.4 mg/g total solids per day and K_m values of 152 mg/l were achieved. The impact of 2-CP on kinetic parameters of glucose degradation by 2-CP degrading granules (adapted biomass) in comparison with glucose-degrading granules (non-adapted biomass) were also determined. It was found that 2-CP caused a mixed inhibition of glucose degradation by adapted and non-adapted biomass. The high Km values of adapted biomass indicated that the degradation of glucose by this biomass was strongly dependent on the glucose concentration.

To evaluate the degradation potential of 2-CP degrading granules for other chlorophenols, these were supplied in the presence and absence of electron donors (e.g. glucose, ethanol, lactate, formate and H_2 gas). The cultures were able to remove chlorine substituents at *ortho*, *meta* and *para* position. All tested monochlorophenols, dichlorophenols and trichlorophenols, except 3,4-DCP and 2,4,5-TCP, were completely dechlorinated and were mineralized to methane and carbon dioxide when glucose was used as electron donor. The selected granules

appeared to have sufficient endogenous reductants to support extensive reductive dechlorination, as samples with no addition of external electron donors could dechlorinate all examined chlorophenols over the incubation period. When compared with non-amended samples, it is apparently that most of the electron donors other than glucose did not enhance the dechlorination rate or transformation efficiency of these cultures. In addition, no relationship between the position of chlorine substituents and the effect of each examined electron donor on transformation of chlorophenols was found.

The 2-CP-degrading granules had also shown the potential of transforming PCP both in the presence and absence of electron donors although approx. 30 % of disappeared PCP was found to adsorb to the granules and presumably glass walls. The experimented results of PCP biosorption by 2-CP-degrading granules fitted well to a Freundlich equation with the parameter, K = 5.5 x 10^{-2} and $1/n$ = 0.97. Glucose seemed to be more effective in increasing the extent of PCP transformation than other electron donors, since many less-chlorinated phenolic intermediates including phenol were detected in PCP samples amended with glucose.

ZUSAMMENFASSUNG

Chlorphenole finden in der Industrie wegen ihrer insektiziden und germiziden Wirkung vor allem als Holzschutzmittel Verwendung und tragen auf diese Weise zu der Verseuchung zahlreicher Böden und Grundwässer bei. Der Abbau von Chlorphenolen erfolgt in der Natur durch photochemische Reaktion bzw. biologische Dehalogenierung. Anaerobe Prozesse erwiesen sich für die Dechlorierung der monosubstituierten und mehrfachsubstituierten Chlorphenole als geeignete Verfahren, während aerobe Systeme mehr für den biologischen Abbauprozess von monosubstituierten Chlorphenolen geeignet sind.

Die 2-CP-abbauende Kultur in dieser Arbeit wurde durch die Adaptation einer pelletierten glucose-abbauenden Biomasse an 2-Chlorphenol (2-CP) etabliert. Diese Kultur wurde weiterhin bei kontinuierlicher Zugabe von 0.9 g/l Glucose als Co-Substrat und steigendem Anteil an 2-CP im labormaßstäblichen Upflow Anaerobic Sludge Blanket (UASB) Reaktor bei einer Temperatur von 35°C angereichert. Physiologische und biologische Faktoren, die die biologische Dehalogenierung durch die adaptierte Kultur beeinflussen (z.B. Anfangs-konzentration des 2-CP, pH des Mediums, Einfluß des organischen Substrates) wurden bestimmt. Für den Reaktorbetrieb wurden folgende Ergebnisse erhalten :

- am Ende des Betriebs konnte die 2-CP-Belastung von 0.6 g/l, d bei einer Verweilzeit von

  1.6 Tagen und einer 2-CP Abbauleistung von über 90 % erreicht werden.

- die 2-CP-abbauende Kultur konnte 2-CP sowohl im UASB-Reaktor als auch im Batch-Versuch mit/ohne Glucose als Co-Substrat zur Methan und Kohlendioxid mineralisieren.
- eine spezifische Dechlorierungsrate bis zu 66.4 mg /g TS, d bei einem K_m-Wert von 152 mg/l wurde erreicht.

Die Wirkung des 2-CPs auf dem Glucoseabbau der 2-CP-abbauenden Kultur wurde bestimmt. Die Auswertung ergab einen gemischten Hemmtyp.

Zur Bestimmung des Abbaupotentials der 2-CP-abbauenden Kultur für andere Chlorphenole wurde die 2-CP-abbauende Biomasse mit verschiedenen Chlorphenolen mit und ohne Zugabe von E'-Donatoren (z.B. Glucose, Äthanol, Lactat, Formiat, H_2-Gas) inkubiert. Die angereicherte Kultur konnte die Chlorsubstituenten in *ortho*-, *meta*- und *para*-Stellung dehalogenieren. Alle getesten Monochlorphenole, Dichlorphenole und Trichlorphenole, ausgenommen 3,4-DCP und 2,4,5-TCP, wurden vollständig dechloriert und mineralisiert, wenn Glucose als E'-Donor verwendet wurde. Die Anreicherungskultur hatte auch genügend endogenes Reservematerial für die reduktive Dechlorierung, da alle getesten Chlorphenole in den Ansätzen ohne E'-Donor-Zusatz innerhalb des gemessenen Zeitraums dechloriert werden konnten. Andere E'-Donatoren fördern weder die Dechlorierungsrate noch die Umsatzrate dieser Kultur im Vergleich zu den mit Glucose gemessenen Raten. Außerdem wurde keine Abhängigkeit zwischen der Stellung der Chlorsubstituenten und dem getesten E'-Donor gefunden.

Die 2-CP-abbauende Kultur zeigte auch ein Potential zur Umsetzung von PCP, sowohl in den Ansätzen mit E'-Donor-Zusatz als auch in denen ohne E'-Donor-Zusatz. Ca. 30 % des verschwundenen PCP war an Biomasse und vermutlich an den Flaschenwänden adsorbiert. Die experimentiellen Ergebnisse der Biosorption von PCP durch die 2-CP-abbauende Kultur können mit einer Freundlich Isotherme beschrieben werden, mit den Parametern K = 5.5 x 10^{-2} und $1/n$ = 0.97. Glucose hatte mehr Wirkung auf die Erhöhung der Biotransformation des PCPs als andere E'-Donatoren. Es wurden verschiedene Dechlorierungsprodukte einschließlich von Phenol in den Ansätzen mit Glucose-Zusatz detektiert.

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