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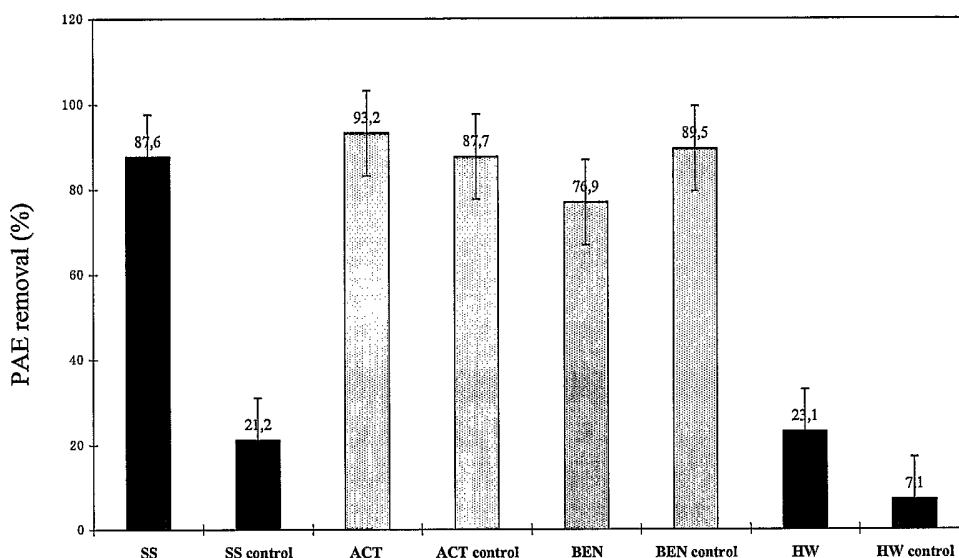
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(54) Title: ANAEROBIC MICROBIAL DEGRADATION OF PHTHALIC ACID ESTERS



(57) Abstract: The disclosed invention relate to a process for anaerobic microbial degradation of phthalic acid esters. Further the invention disclosed a method for isolating bacterial strains, which strains in pure form each are capable of anaerobic degradation of phthalic acid esters. The invention further relate to isolated bacterial strains capable of anaerobic phthalic acid ester degradation.

Title: Anaerobic microbial degradation of phthalic acid esters

Technical Field

The present invention relates to a process for anaerobic microbial degradation of
5 phthalic acid esters, to a method for isolating bacterial strains capable of anaerobic
degradation of phthalic acid esters as pure bacterial strains, and to the use of such
strains in anaerobic biological systems.

Background Art

10 Disposal of sewage sludge is a growing problem in Europe due to the increasing
amounts of sludge produced every year. More than 9 millions tons of dry solids are
expected to be produced in 2005, and around 40% of this amount is currently reused
as fertilisers on agricultural soils. However, the sludge may contain hazardous chemi-
cals in significant amount making their reuse on land problematic. Man-made organic
15 chemicals (xenobiotics) are especially found in wastewater treatment plants, and read-
ily accumulate in sludge since most of them present hydrophobic properties. In order
to ensure a safety reuse of sludge on land, the EU regulations related to the xenobiotic
contents are continuously reinforced. Therefore, the treatment of the organic pollutants
before disposal, especially by anaerobic digestion which is the most commonly applied
20 method for biological sludge treatment, has become of increasing interest over the last
ten years at a societal, policy and research level.

The phthalic acid esters (PAEs) are specifically concerned by EU regulations because
of the increasing amounts released in the environment. Last year, more than 900,000
25 tons of PAEs were produced in Europe (ECPI, 2004). Most of the PAE's like Di-
Methyl-Phthalate ester (DMP), Di-Butyl-Phthalate ester (DBP), Di-Ethyl-Phthalate ester
(DEP), Benzyl-Butyl-Phthalate ester (BBP), Di-Ethyl-Hexyl-Phthalate ester (DEHP) are
used as plasticizers to increase the flexibility of the polyvinylchloride (PVC) resins, and
also as additives in other resins such as polyvinyl acetates, celluloses, and polyure-
30 thanes (Staples, C.A., Peterson, D.R., Parkerton, T.F., and W.J. Adams. 1997. The en-
vironmental fate of phthalate esters: a literature review. Chemosphere. 35:667-749).
Amongst these, the DBP and DEHP are utilized as additive in epoxy resins, cellulose
esters and specialized adhesive formulations, and also as a solvent for many dyes, in-
secticides and other organic compounds (Staples *et al.*, 1997; ECPI, 2004). Since
35 DBP and DEHP are surface active and hydrophobic compounds, they readily adsorb
onto sludge organic particles during wastewater clarification. The DBP and DEHP

- therefore up-concentrate to orders of magnitude above the values in the original wastewater, reaching levels higher than 600 mg/kg_{DW} in sewage sludge. By spreading untreated sludge, the DBP or DEHP may not only accumulate in soils (Hu, X.Y., Wen, B., and X.Q. Shan. 2003. Survey of phthalate pollution in arable soils in China. *J. Environ. Monit.* 5:649-653), but could also readily transfer to plants (Yin, R., Lin, X.G., Wang, S.G., and H.Y. Zhang. 2003. Effect of DBP/DEHP in vegetable planted soil on the quality of capsicum fruit. *Chemosphere*. 50:801-805). Moreover, a long term or a high exposure of human to DBP or DEHP can adversely affect human reproduction or development (CEHR, Center for the Evaluation of risks to Human Reproduction. 2000. National Toxicology Program – CEHR monograph on the potential human reproductive and developmental effects of di-n-butyl phthalate (DBP). [Online.] <http://cerhr.niehs.nih.gov>). Therefore, limitation of DBP or DEHP contents in sludge is strongly recommended before land disposal.
- Many studies have been conducted to estimate the DBP biodegradability under aerobic (Chang, B.V., Yang, C.M., Cheng, C.H., and S.Y. Yuan. 2004. Biodegradation of phthalate esters by two bacteria strains. *Chemosphere* 55:533-538; Angelidaki, I., Mogenssen, A.S., and B.K. Ahring. 2000. Degradation of organic contaminants found in organic waste. *Biodegradation* 11:377-383; Yuan, S.Y., Liu, C., Liao, C.S., and B.V. Chang. 2002. Occurrence and microbial degradation of phthalate esters in Taiwan river sediments. *Chemosphere* 49:1295-1299), denitrifying (Benckriser, G., and J.C.G. Ottow. 1982. Metabolism of the plasticizer di-n-butylphthalate by *Pseudomonas pseudoalcaligenes* under anaerobic conditions, with nitrate as the only electron acceptor. *Appl. Environ. Microbiol.* 44:576-578), sulfate-reducing (Chauret, C., Iniss, W.E., and C.I. Mayfield. 1996. Biotransformation at 10°C of di-n-butyl phthalate in subsurface microcosms. *Ground water* 34:791-794) and methanogenic conditions (Angelidaki, I., Mogenssen, A.S., and B.K. Ahring. 2000. Degradation of organic contaminants found in organic waste. *Biodegradation* 11:377-383; Wang, J., Chen, L., Shi, H., and Y. Qian. 2000. Microbial degradation of phthalic acid esters under anaerobic digestion of sludge, *Chemosphere* 41:1245-1248; Gavala, H.N., Alatrisme-Mondragon, F., Iranpour, R., and B.K. Ahring. 2003. Biodegradation of phthalate esters during the mesophilic anaerobic digestion of sludge. *Chemosphere* 52:673-682). Whatever the conditions, the DBP is described as one of the easiest phthalic acid ester to be degraded because of the shortness of the alkyl branching chain (Chang, B.V., Yang, C.M., Cheng, C.H., and S.Y. Yuan. 2004. Biodegradation of phthalate esters by two bacterial strains. *Chemosphere* 55:533-538; Gavala, H.N., Alatrisme-Mondragon, F., Iranpour, R., and

B.K. Ahring. 2003. Biodegradation of phthalate esters during the mesophilic anaerobic digestion of sludge. *Chemosphere* 52:673-682; Yuan, S.Y., Liu, C., Liao, C.S., and B.V. Chang. 2002. Occurrence and microbial degradation of phthalate esters in Taiwan river sediments. *Chemosphere* 49:1295-1299). However, anaerobic conditions remain less
5 favourable to DBP degradation than aerobic conditions with biodegradation rates up to ten times lower (Yuan, S.Y., Liu, C., Liao, C.S., and B.V. Chang. 2002. Occurrence and microbial degradation of phthalate esters in Taiwan river sediments. *Chemosphere* 49:1295-1299; Staples, C.A., Peterson, D.R., Parkerton, T.F., and W.J. Adams 1997. The environmental fate of phthalate esters: a literature review. *Chemosphere* 35:667-
10 749).

A probable toxic effect was suggested by Angelidaki *et al.* (2000) (Angelidaki, I., Mogenssen, A.S., and B.K. Ahring. 2000. Degradation of organic contaminants found in organic waste, *Biodegradation* 11:377-383) who reported a significant inhibition of the
15 methanogenesis by addition of 200 mg/l of DBP. Some other authors contrarily observed no toxic effect of DBP on methanogenic activity at concentration up to 300 mg/l, likely due to the operating conditions (Gavala, H.N., Alatrisme-Mondragon, F., Iranpour, R., and B.K. Ahring. 2003. Biodegradation of phthalate esters during the mesophilic anaerobic digestion of sludge. *Chemosphere* 52:673-682; O'Connor, O.A., Rivera,
20 M.D., and L.Y. Young. 1989. Toxicity and biodegradation of phthalic acid esters under methanogenic conditions. *Environ.Toxicol.Chem.* 8:569-576). Furthermore, a general anaerobic biodegradation pathway has been proposed by Staples *et al.* (1997) (Staples, C.A., Peterson, D.R., Parkerton, T.F., and W.J. Adams. 1997. The environmental fate of phthalate esters: a literature review. *Chemosphere* 35:667-749). This pathway
25 suggests a first hydrolytic step of one ester bond to form a monoester phthalate and a corresponding alcohol. The second ester bond would also be hydrolysed to lead to the formation of a phthalic acid isomer. The anaerobic degradation of the phthalic acid by methanogenic consortia via the benzoate degradation pathway is then possible, as previously shown (Kleerebezem, R., Hulshoff Pol, L.W., and G. Lettinga. 1999. Anaerobic degradation of phthalate isomers by methanogenic consortia. *Appl. Environ. Microbiol.* 65:1152-1160; Kleerebezem, R., Hulshoff Pol, L.W., and G. Lettinga. 1999. The role of benzoate in anaerobic degradation of terephthalate. *Appl. Environ. Microbiol.* 65:1161-1167; Qiu, Y.L., Sekiguchi, Y., Imachi, H., Kamagata, Y., Tseng, I.C., Cheng, S.S., Ohashi, A., and H. Harada. 2004. Identification and isolation of anaerobic,
35 syntrophic phthalate isomer-degrading microbes from methanogenic sludges treating wastewater from terephthalate manufacturing. *Appl. Environ. Microbiol.* 70:1617-1626).

Although DBP-degrading bacteria have already been isolated and characterized under aerobic conditions (Chang, B.V., Yang, C.M., Cheng, C.H., and S.Y. Yuan. 2004. Biodegradation of phthalate esters by two bacteria strains. *Chemosphere* 55:533-538), only little is known about the microorganisms involved in such DBP biodegradation under anaerobic conditions. Despite the evidence of an efficient DBP biodegradation under methanogenic conditions (Angelidaki, I., Mogensen, A.S., and B.K. Ahring. 2000. Degradation of organic contaminants found in organic waste. *Biodegradation* 11:377-383; Ejlerthsson, J., Meyerson, U., and B.H. Svensson. 1996. Anaerobic degradation of phthalic acid esters during digestion of municipal solid waste under land filling conditions. *Biodegradation* 7:345-352; Gavala, H.N., Alatraste-Mondragon, F., Iranpour, R., and B.K. Ahring. 2003. Biodegradation of phthalate esters during the mesophilic anaerobic digestion of sludge, *Chemosphere* 52:673-682; O'Connor, O.A., Rivera, M.D., and L.Y. Young. 1989. Toxicity and biodegradation of phthalic acid esters under methanogenic conditions. *Environ.Toxicol.Chem.* 8:569-576), the isolation of pure anaerobic bacteria capable of PAE degradation is hardened by the need of complex syntrophic relationship in anaerobic environment, as previously shown by Qiu et al. (2004) (Qiu, Y.L., Sekiguchi, Y., Imachi, H., Kamagata, Y., Tseng, I.C., Cheng, S.S., Ohashi, A., and H. Harada. 2004. Identification and isolation of anaerobic, syntrophic phthalate isomer-degrading microbes from methanogenic sludges treating wastewater from terephthalate manufacturing. *Appl. Environ. Microbiol.* 70:1617-1626).

In addition, anaerobic degradation of sludge is much more preferable than aerobic degradation due to lower costs of maintenance and reuse of biogas energy, and is widely used all over the world in WasteWater Treatment Plants (WWTP). Therefore, the enhancement of the PAE-degrading potential of anaerobic ecosystems treating sludge by adding enrichment cultures or pure isolates of bacteria, as pure cultures or mixed cultures, capable of efficient anaerobic degradation of PAEs would be very desirable.

Disclosure of Invention

By an especially designed enrichment and isolation method, enriched cultures based on natural isolates as well as pure isolated strains which are very efficient for the anaerobic degradation of phthalic acid esters from such cultures have now been obtained which are very efficient for the anaerobic degradation of phthalic acid esters.

In a first aspect the present invention relates to a process for anaerobic microbial degradation of phthalic acid esters, comprising the step of adding to a bioreactor at least

one bacterial strain, which as a pure isolate is capable of anaerobic degradation of phthalic acid esters.

In a second aspect the present invention relates to a method for isolating bacterial strains, which strains in pure form each are capable of anaerobic degradation of phthalic acid esters, comprising the steps of:

a) enrichment for bacterial populations comprising bacterial strains capable of anaerobic degradation of phthalic acid esters as pure isolated strains, comprising the cultivation of natural bacterial isolates under anaerobic conditions in the presence of phthalic acid esters under CSTR conditions;

b) isolation of single colonies on nutrient plates comprising phthalic acid esters.

In a third aspect the present invention relate to an isolated bacterial strain capable of anaerobic phthalic acid ester degradation and said strain being obtainable by the method according to the invention, wherein said isolated bacterial strain is capable of anaerobic degradation of at least 40 % of PAE present in the culture medium at concentration up to 500 mg/l, particularly at least 50 %, more particularly at least 60 %, when the said degradation is performed by the pure isolate of said bacterial strain either alone or in a defined culture of isolated strains.

In a fourth aspect the present invention relates to an isolated bacterial strain capable of anaerobic degradation of phthalic acid esters selected from the group consisting of DSM deposit numbers DSM 17351, DSM 17352, DSM 17353 and DSM 17354

In a fifth aspect the present invention relates to a composition comprising at least one isolated strain according to the invention.

Brief Description of the Drawings

The invention is explained in detail below with reference to the drawings, in which

Fig. 1 shows PAE removal in control and biological sludge-inoculated reactors (SS: sewage sludge, ACT: activated carbon 10g/L, BEN: Bentonite (10g/L) and HW =

Household Waste digested sludge). Controls correspond to chemically sterilized reactors by addition of 2% formaldehyde and 1,4% sodium azide.

Fig. 2 shows the impact of semi-continuous (Fed-Batch) and continuous (CSTR) enrichment procedures on specific PAE (DBP) removal rate.

Fig. 3 shows the ability of enriched and non-enriched cultures to degrade DBP and DEHP.

Fig. 4 shows the effect of increasing initial DBP concentration on DBP removal in the presence of an additional carbon source, by enriched culture (A) and by non-enriched culture (B).

Fig. 5 shows the effect of the addition of enrichment culture to a low-PAE-degrading sludge (HW : household-waste-digesting anaerobic sludge). The control corresponds to a chemically sterilized reactor.

Fig. 6 shows phthalic Acid Ester (DBP) removal after 2 days of batch cultivation under anaerobic conditions. The culture medium correspond to a mixture of BA medium, 2g/L yeast extract and 30 mg/l DBP. CTA and CTB correspond to control reactors (non inoculated).

Fig. 7 shows phthalic Acid Ester (DEHP) removal after 2 days of batch cultivation under anaerobic conditions. The culture medium correspond to a mixture of BA medium, 2g/L yeast extract and 50 mg/l DBP. CTA and CTB correspond to control reactors (non inoculated).

Fig 8 shows the phylogenetic tree of the related microorganisms close to the four PAE-degrading isolated bacteria (DSMZ accession numbers: DSM 17351, DSM 17352, DSM 17353 and DSM 17354. The tree was generated using ARB fast aligner for sequence alignment within *E.coli* positions and by the neighbour joining distance method using Felstensteins PHYLIP algorithm in ARB software, with *Escherichia coli* as the root outgroup. Bar indicates Jukes-Cantor evolutionary distance.

Fig. 9. Probe sequences targeting eubacteria and archaeobacteria suspected to be involved in PAE degradation pathway. Blank probes target general dominant bacteria in

sludge reactor. Non group hit number correspond to probe targeted sequences presenting 100% similarity but not belonging to the target group/species, according to the NCBI database (<http://www.ncbi.nlm.nih.gov/>).

5 Fig. 10 shows the impact of different mixtures of the isolates on DBP removal.

Fig.11 shows the impact of different mixtures of the isolates on DEHP removal.

Fig. 12 shows the global strategy for Single Strand Conformation Polymorphism
10 (SSCP)

Fig. 13 shows Archaeal (a) and Bacterial (b) SSCP Profiles at steady state (0 – 10 – 200 mg/l Phthalic Acid Ester)

15 Fig. 14 shows relative abundance of SSCP peaks in blank reactor (eubacteria).

Fig. 15 shows relative abundance of SSCP peaks in 10mg/L-PAE reactor (eubacteria).

Fig. 16 shows relative abundance of SSCP peaks 200mg/L-PAE reactor (eubacteria)
20

Fig. 17 shows relative abundance of SSCP peaks in blank reactor (archaeobacteria)

Fig. 18 shows relative abundance of SSCP peaks in 10mg/L-PAE reactor (archaeobacteria).
25

Fig. 19 shows relative abundance of SSCP peaks in 200mg/L-PAE reactor (eubacteria)

Fig. 20 shows a phylogenetic tree of the dominant eubacterial species identified from non-enriched (Bact4 and Bact5) and PAE enriched cultures (Bact1, Bact2 and Bact3)
30 (in bold). The tree was generated using ARB fast aligner for sequence alignment within *E.coli* positions and by the neighbour joining distance method using Felstensteins PHYLIP algorithm in ARB software, with *E.coli* 206 as the outgroup. Numbers at nodes indicate percent bootstrap values (1000 replicates). Bar indicates Jukes-Cantor evolutionary distance.

35

Fig. 21 shows a phylogenetic tree of the dominant archaeobacteria species identified from non-enriched (Arch3) and PAE enriched cultures (Arch4) (in bold). The tree was generated using ARB fast aligner for sequence alignment within *E.coli* positions and by the neighbour joining distance method using Felstensteins PHYLIP algorithm in ARB software, with *Methanosarcina mazeii* as the outgroup. Numbers at nodes indicate percent bootstrap values (1000 replicates). Bar indicates Jukes-Cantor evolutionary distance.

Best Modes for Carrying out the Invention

10 The present invention is based on the finding that it is possible to provide cultures of specially adapted micro organisms capable of anaerobic degradation of phthalic acid esters. These cultures are based on micro organisms found in environmental ecosystems, e.g. sludge, and subsequently grown and selected by the method of the present invention in order to provide said enriched cultures as described herein. From this method, it is possible to isolate pure novel bacterial strains which are capable of growing both anaerobically and aerobically on PAEs. The isolated strains according to the invention, in the form of pure cultures of each strain or as any combinations of the pure strains, herein known as defined cultures, are capable of anaerobic degradation of phthalic acid esters. These novel strains therefore have a great potential as additives for conventional anaerobic bioreactors for treating sludge or equivalent material.

One aspect of the invention therefore relates to a method of enrichment for bacterial populations comprising bacterial strains capable of anaerobic degradation of phthalic acid esters as pure isolated strains, comprising the cultivation of natural bacterial isolates from sludge under anaerobic conditions in the presence of phthalic acid esters.

The potential for the selection or enrichment to result in the optimal bacterial composition of mixed cultures displaying the most efficient ability to degrade phthalic acid esters depends on several factors. The starting bacterial flora can be obtained from different sources of sludge or other waste material, like e.g. sewage water, sludge, waste, household waste, refuse, or liquid or semi-liquid manure.

In the present invention the term "sludge or other waste material" should be understood as any waste material having a high content of organic matter of at least 40% such as e.g. in the range from 50 to 80 %, and a total solids content from 1 to 6 %.

In a particular embodiment the sludge is sewage sludge or sewage water. Other relevant types of waste material could be waste, e.g. household waste, refuse, or liquid or semi-liquid manure.

- 5 Sewage sludge has been shown in the present invention to possess a suitable starting flora for the enrichment method and subsequent isolation of pure bacterial strains.

The success of the enrichment method according to the invention also depends on the type of reactor used for the enrichment. Two different enrichment procedures were
10 tested and compared according to their potential for enhancing PAE removal efficiency: one series of successive Fed-batch reactors, and one continuous stirred tank reactor, CSTR. For each, one abiotic control - chemically sterilized – representing the abiotic losses, and one population control - a blank without xenobiotic addition – were performed. In order to enrich the microbial ecosystems in PAE-degrading microorganisms,
15 the reactors were fed with a specific anaerobic synthetic medium providing vitamins and cofactors needed for the growth of anaerobic bacteria (BA medium). Yeast Extract and Di-Butyl-Phthalate ester (DBP – 10mg/L) were also added as, respectively, nitrogen/phosphorus and PAE sources.

- 20 The results showed that the biological xenobiotic-degrading ability of the ecosystem was significantly enhanced in all of the reactors (see example 1). However, the continuous process in a continuous stirred tank reactor (CSTR) turned out to be the most efficient procedure for enhancing the PAE biodegradation in comparison with successive series of Fed-batch reactors.

25

The enrichment was carried out for at least 40 days, particularly for at least 60 days and more particularly for at least 80 days in successive fed batch reactors. For the CSTR, the enrichment was carried out for at least two hydraulic retention times, more particularly for at least three hydraulic retention times, and even more particularly for at
30 least four hydraulic retention times.

As described herein the term “hydraulic retention time” means the average time that a single particle (or bacterium) stays in the reactor. The Hydraulic Retention Time(s) thus refer to how long a given material is kept (retained) in the liquid system.

35

The phthalic acid ester present in the culture medium during the enrichment can be any phthalic acid ester, like e.g. Di-Butyl-Phthalate (DBP), Di-Methyl-Phthalate (DMP), Di-Ethyl-Phthalate (DEP), Di-Ethyl-Hexyl-Phthalate (DEHP), Benzyl-Butyl-Phthalate ester (BBP), and particularly the phthalic acid ester is selected from the group consisting of

5 Di-Butyl-Phthalate (DBP) or Di-Methyl-Phthalate (DMP). The enriched cultures or the isolated pure bacterial strains, which can be isolated there from, are capable of degrading all phthalic acid ester also known as plasticizers, however, for the enrichment particularly such PAEs which are not toxic for the bacterial culture should be used. These include e.g. DBP and DMP. These are good choices since they are readily de-

10 graded and do not interfere with bioaugmentation.

The term "degradation" as used herein should mean biological transformation of the initial contaminant. It includes complete or partial mineralization of the molecule, as well as a single oxidation or carboxylation of the molecule.

15 When DBP is used in the culture medium the concentration of DBP is applied in the range from 2-1000 mg/l in the reactor, particularly from 5-500 mg/l, more particularly from 10-300 mg/l, even more particularly from 20-100 mg/l.

Cultures enriched according to the above described method will possess unique

20 phthalic acid ester degrading properties. Such cultures will be capable of efficient anaerobic degradation of phthalic acid esters in the form of defined cultures based on pure isolates. Pure isolates can be used alone or in combination. Such combinations of cultures are in the present invention termed defined cultures.

25 Since the anaerobic sludge has been enriched for its PAE degrading property it has now been possible to isolate for the first time pure bacterial strains, which strains each are capable of anaerobic degradation of PAEs.

In a further aspect the invention therefore relates to a method for isolating bacterial

30 strains, which strains in pure form each are capable of anaerobic degradation of phthalic acid esters, comprising the steps of:

a) enrichment for bacterial populations comprising bacterial strains capable of anaerobic degradation of phthalic acid esters as pure isolated strains, comprising the cultivation of natural bacterial isolates under anaerobic conditions in the presence of phthalic

35 acid esters under CSTR conditions;

b) isolation of single colonies on nutrient plates comprising phthalic acid esters.

In one embodiment the plates are BA-minimal agar plates containing PAE (10 to 500
5 mg/ml) as sole carbon source.

In one particular embodiment the invention relates to an isolated bacterial strain capable of anaerobic phthalic acid ester degradation and being obtainable by the method according to the invention, wherein said isolated bacterial strain is capable of anaerobic
10 degradation of at least 40 % of PAE present in the culture medium at concentration up to 500 mg/l, particularly at least 50 %, more particularly at least 60 %, when the said degradation is performed by the pure isolate of said bacterial strain either alone or in a defined culture of isolated strains.

15 Preferably, the bacterial isolates according to the invention are capable of growth also in aerobic conditions. Thereby it is possible to build up biomass in a convenient way, prior to use in bioaugmentation.

The enriched cultures according to the invention comprises bacterial strains, which in
20 their pure form as isolated strains are capable of anaerobic degradation of phthalic acid esters. Such strains can be isolated as described above and as illustrated in example 3 below, e.g. based on successive selections on an anaerobic synthetic medium and phthalic acid ester, e.g. DBP, as the carbon source.

25 Preferably, the enrichment cultures according to the invention are essentially capable of growth also in aerobic conditions. Thereby it is possible to build up biomass in a convenient way, prior to use in bioaugmentation.

The enrichment protocol and the subsequent isolation of pure isolated bacterial strains
30 resulted in four bacterial strains which were characterized as described in the examples below, and their PAE degrading properties were investigated. Each isolate exhibited a different removal performance from around 50% to more than 90%.

The four bacterial strain were deposited, on 4th May in the DSMZ institute (DSMZ-
35 Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
GmbH Mascheroder Weg 1b, D-38124 Braunschweig) in accordance with the Buda-

pest treaty related to the deposit of microorganisms for the purpose of a patent procedure. The DSMZ accession numbers are the following: DSM 17351, DSM 17352, DSM 17353 and DSM 17354

- 5 In one further aspect the present invention therefore relates to an isolated bacterial strain capable of anaerobic degradation of phthalic acid esters selected from the group consisting of DSMZ accession numbers DSM 17351, DSM 17352, DSM 17353 and DSM 17354.
- 10 The isolated bacteria are suitable as an additive for an anaerobic bioreactor either as separate strains/cultures or as combinations of the four strains.

In one embodiment the strain(s) is comprised in the enriched culture. In another embodiment the isolated strains are in the form of a defined culture consisting of one or
15 more of the isolated strains.

The strains can be in the form of a composition comprising at least one of the above strains or any other pure isolate obtainable by the method of the invention. In one embodiment the composition can be in the form of a powder, like a lyophilized, freeze
20 dried or dried powder.

The new isolates described above as well as enriched cultures produced by the method of the invention can advantageously be added to conventional anaerobic bioreactors in order to improve the capacity for removing PAEs from sludge.
25

- Though the enriched cultures as such can be applied for the removal of PAE, the defined cultures comprising one or more of the isolated pure strains according to the invention will result in the most efficient removal of PAE.
- 30 One aspect of the invention therefore relates to a process for anaerobic microbial degradation of phthalic acid esters, comprising the step of adding to a bioreactor at least one bacterial strain, which strain as a pure isolate is capable of anaerobic degradation of phthalic acid esters.

In a particular embodiment the phthalic acid esters are comprised in sewage water, sludge, waste, household waste, refuse, or liquid or semi-liquid manure or other waste material.

- 5 In a further embodiment the at least one bacterial strain capable of anaerobic degradation of phthalic acid esters as a pure isolate is comprised in an enriched culture or a defined culture according to the invention. The enriched culture or the defined culture can be added once or continuously, i.e. at least once a quarter, particularly once a month, more particularly every second week, even more particularly every week. When the
- 10 culture is added continuously it is in a particular embodiment added in an amount of at least 5 % v/v, particularly at least 10 % v/v, more particularly at least 15 % v/v, even more particularly at least 20 % v/v, and the added culture comprises from 10^2 - 10^9 bacteria/ml, more particularly from 10^4 - 10^6 bacteria/ml

- 15 The pure or defined culture can advantageously be grown under aerobic conditions before addition to the bioreactor in order to facilitate the growth of the culture.

The enriched culture according to the invention comprises in one embodiment at least one bacterial strain according to the invention, particularly at least two bacterial strains,

20 more particularly at least three bacterial strains, such as e.g. all four bacterial strains, which in pure form each are capable of anaerobic degradation of phthalic acid esters. In case the culture comprises only pure isolates the culture is termed a defined culture.

The bacterial strains which in pure form each are capable of anaerobic degradation of

25 phthalic acid esters are particularly selected from the phylogenetic groups consisting of the genera *Exiguobacterium* and *Bacillus*.

The *Exiguobacterium* comprises, in a particular embodiment *Exiguobacterium gaetbuli* strains. More particularly said strains are selected from the group consisting of DSMZ

30 deposit number *Exiguobacterium* sp. DBPA, DSM 17351 and DSMZ deposit number *Exiguobacterium* sp. DBPB, DSM 17352.

The *Bacillus* according to the invention comprises *Bacillus fusiformis* and *Bacillus licheniformis* strains. Particularly the *Bacillus fusiformis* strain is DSMZ deposit

35 sp. DBPC DSM 17353, and the *Bacillus licheniformis* strain is DSMZ deposit number *Bacillus* sp. DBPD DSM 17354,

The invention is further illustrated by the following specific examples.

Examples

5

Materials and methods

Reactor design & microbial ecosystems

- 10 Reactor design: Three different types of reactors were implemented to enrich and characterize the anaerobic microbial communities degrading xenobiotics. (i) Batch reactors were performed in 100ml flasks containing 2/3 of anaerobic spiked BA medium (see below) and 1/3 of anaerobic microbial ecosystem that needed to be tested (digested sludge or enrichment culture). The batch experiments were conducted during
- 15 15 days. Batch pre-cultures of isolated bacteria were performed in 5ml, and were used for further inoculation of 95ml spiked BA medium. The batch experiments using pure isolate precultures as inocula were performed within maximum one week (ii) Fed-batch reactors were used to enrich xenobiotic degraders within complex microbial population. They were performed in 500 ml flasks with an initial volume of 300 ml (2/3 BA medium
- 20 and 1/3 digested sludge). Every four days, 10% of the total volume in spiked BA medium was added to the reactor up to full filling of the flask. Then, 200 ml of the enrichment culture were added to 100mL of fresh spiked BA medium, and transferred to a new Fed-batch reactor (2nd cycle). Repetitively, up to four cycles of enrichment were performed (iii) CSTR (continuous stirred tank reactors) were also used as an alternative enrichment technique. They were performed in 500ml flasks. Starting inoculum
- 25 was a mixture of 1/3 of spiked BA medium and 2/3 of digested sludge. The reactors were fed daily and manually with spiked BA medium. Reactor inlet corresponded to the same volume of sample taken in the outlet (no accumulation of liquid in the reactor). The hydraulic retention time of the reactors was 20 days, and reactors were kept running for at least four hydraulic retention times.
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At start, all reactors were flushed with a mixture of N₂:CO₂ (80:20 v:v) to set the reactors under anaerobic conditions. Then, they were incubated at 37°C under magnetic stirring conditions.

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Microbial ecosystems: Biological inocula were first chosen for their highly probable ability to degrade anaerobically xenobiotics due to their natural environment, i.e. long term and high-level xenobiotic contamination. The PAE inoculum corresponded to an anaerobic digested sludge of a waste water treatment plant WWTP contaminated by Phthalic Acid Esters for a long period (Lynetten, Denmark).

Anaerobic BA medium: The cultivation medium was previously optimized in the host laboratory by Assoc. Prof. I. Angelidaki *et al.* (partially available in literature). This medium was partially modified for the present study and corresponded to a mixture of macronutrients [(g/L) NH_4Cl , 1; NaCl , 0.1; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1; $\text{CaCl}_2 \cdot \text{O}$, 0.05; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.4], trace metals [(mg/L) $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 2; H_3BO_3 , 0.05; ZnCl_2 , 0.05; CuCl_2 , 0.03; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.05; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.05; AlCl_3 , 0.05; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05; NiCl_2 , 0.05; EDTA, 0.5; $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$, 0.1], NaHCO_3 (2,6 g/L), $\text{Na}_2\text{S} \cdot 7-9\text{H}_2\text{O}$ (0,25 g/L) and vitamins [(mg/L) Biotin, 2; Folic acid, 2; Pyridoxine, 10; Riboflavin, 5; Thiamine, 5; Cyanocobalamin, 0.1; Nicotinic acid, 5; P-amino-benzoic acid, 5; Lipoic acid, 5; DL-panthothenic acid, 5]. BA medium was then supplemented with 2 g/L of Yeast Extract providing nitrogen and phosphorus sources, and with xenobiotics at a concentration ranging from 10 to 500 mg/L (PAE). The latter mixture is called "spiked BA medium".

Molecular biology methods

This section describes the molecular techniques developed and used for characterizing the 16S rDNA sequences of the pure isolates.

Sampling and total genomic DNA extraction from pure isolates: Two-millilitres of liquid samples were periodically prepared by adding colonies from agar plates to BA medium. Each colony grew from 2 to 4 days on agar Petri plates, and was placed in a 0.2 ml PCR microtube (Eppendorf, USA) containing 20 μl of water. The samples were centrifuged at 6000 g (10 min., 4°C). The extraction and the purification of bacterial genomic DNA were performed with QIAamp DNA stool Mini Kit (Quiagen, Hilden, Germany). The products of extraction were identified by electrophoresis on a 0.7 % agarose gel. DNA bands were detected with $5 \cdot 10^{-5}$ % (w/v) ethidium bromide staining and visualized by UV light.

Amplification of the SmallSubUnit rDNA sequence: The total SSU (small sub-unit) rDNA was amplified by PCR with the following couples of primers: Universal reverse

primer R1492, *E.coli* position 1492, from 5' to 3' (GNTACCTTGTTACGACTT), and the bacterial forward primer F9, *E.coli* position 9, from 5' to 3': GAG TTT GAT CMT GGC TCA G). Each reaction tube contained 0.2 µg of each primer, 1.0 µl of purified DNA, 5µl Taq reaction buffer with 25mM MgCl₂, 0.8 mM dNTP and 1.25 U of Taq polymerase
5 (Stratagene, USA), adjusted to a total volume of 50 µl. The reactional tube was then placed in a thermocycler (9600 Perkin-Elmer) at 94°C. For all PCR, three-stage cycles were performed 30 times : 94°C for 1 min., 51°C for 1 min., 72°C for 1 min. and a final stage 72°C for 10 min. The amplification was checked by loading 10% (v/v) of the PCR product on a 2.0% agarose gel. The bands were detected with 5.10⁻⁵ % (w/v) ethidium
10 bromide staining and visualized by UV light. The expected size of the band was around 1500 bp lengths, and was checked using a 150 bp DNA ladder (Promega, Wis., USA).

Sequencing of SSU rDNA genes from clones: The PCR products were further sequenced using the dideoxy-chain-termination method, and the ABI model 373A sequencer apparatus (Applied Biosystems). Plasmid DNA was sequenced with the dye-terminator cycle-sequencing reaction kit with AmpliTaq DNA polymerase FS kit buffer (Perkin-Elmer) and the pGEMt primers T7 and P13. A partial sequence of 1500 bp, corresponding to the 16S rDNA was determined for each pure isolate.

20 Phylogenetic analysis: An equal portion of SSU rDNA (*E.coli* position 9 to 1492 / ~1500pb) was used for the sequence analysis. Sequences from clones were submitted to Genbank for preliminary analysis using the Blast program of the Ribosomal Database Project to identify putative close phylogenetic relatives. Sequences were aligned to their nearest neighbour with the automated alignment tool of the ARB program
25 package (<http://www.arb-home.de/>). The phylogenetic tree showing relationship of 16S rDNA archaeal clones sequences was generated by neighbour-joining analysis and corrected with a filter that included only close sequences. One thousand bootstrap analyses were performed and percentages greater than 50% were reported on the phylogenetic trees.

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Fluorescence *In Situ* Hybridization (FISH) method

Short introduction of FISH: Fluorescence *in situ* hybridization (FISH) of whole cells using 16S rRNA-targeted oligonucleotide probes is a method for identifying microbes
35 visually in a complex environment. It involves application of oligonucleotide probes to fixed microbial cells. The DNA probe enters the cells and specifically hybridizes to its

complementary target sequence in ribosomal RNA. The probes are typically 5' end-labelled with fluorescent molecule for observation with epifluorescence microscopy.

Procedure of FISH experiment: The method used was that of Hugenholtz *et al.* (2001 –

- 5 Design and evaluation of 16S rRNA-targeted oligonucleotide probes for FISH. In: *Methods in Molecular Biology*, vol.176. Ed. B.A. Lieberman, Totowa, NJ). It consisted of fixation, hybridization, and observation, as follows:

* **Fixation:** Approximately 1ml of sample (more for dilute samples below 500
10 mg/L) was added to a 2 ml microcentrifuge tube, and centrifuged for 2 minutes at 6-10 g. After removing the supernatant, 2 ml of paraformaldehyde was added and the pellet resuspended. The solution was hold for 1-3 hours at 4°C (4-6 hours for granules). The sample was then centrifuged, resuspended in PBS-1x, centrifuged again, and finally resuspended in 1ml of PBS-1x. One ml ice-cold 100% ethanol was then added, and the
15 sample was stored at -20°C until hybridization. Samples can be stored for several months.

* **Hybridization:** Five µl of sample was applied to each well to a Teflon-coated slide, and air-dried. Then, samples were dehydrated in an ethanol series of 50%, 80%,
20 and 98% of ethanol (3 minutes each). The dehydrated slide can be stored at room temperature for approximately 1 month. An aliquot of 2 ml of hybridization buffer was prepared in a 2ml microcentrifuge tube. This buffer should contain 360 µL 5M NaCl, 40 µL HCl, x µL formamide (400 µL for 20% stringency), y µL sterilized MQ water (1198 µL for 20% stringency), 2 µL 10% SDS (add last in the cap, and mix rapidly). The
25 amount of formamide and MQ water (x and y) depend on the stringency to be needed. This depends on the specificity of the probe, and accessibility of the region of ribosome to which the probe targets. Normally, a stringency of 20% is used for general probes. To adjust stringency, the formamide/MQ ratio can be change from 0/1598 for 0% to 1000/598 for 50%. Then, 8µL hybridization buffer and 0.5µL of each probe were ap-
30 plied on each well. The rest of the hybridization buffer was poured onto a paper bed in a 50 ml polypropylene tube. The slide was carefully placed on top of this bed (well-side up), and placed in the hybridization oven set at 46°C for 1-2 hours. The oven was started at least half an hour before hybridizing to stabilize the temperature. When hybridization was finished, the slide was washed using a preheated 48°C wash buffer
35 containing z µL 5M NaCl (2150 mL for 20% formamide, see below for others), 1 mL 1M

Tris-HCl, 50 μ L 10% SDS (in cap, invert, and rapidly mix) and milli-Q water up to 50 mL. All hybridization solution and probe were flushed from the surface of the slide which was then placed in water for 10-15 minutes. The slide was finally mounted (2-3 drops of Citifluor), and observation was performed using a confocal or epifluorescence microscope.

[NaCl z volumes: Formamide (%): NaCl (μ L); 0%:9000 μ L, 5%:6300 μ L, 10%:4500 μ L, 15%:3180 μ L, 20%:2150 μ L, 25%:1490 μ L, 30%:1020 μ L, 35%:700 μ L, 40%:460 μ L, 45%:300 μ L, 50%:180 μ L]

Single Strand Conformation Polymorphism – SSCP technique.

The molecular techniques used in this project were previously developed and optimized at the Laboratory of Environmental Biotechnology (LBE – National Institute of Agronomic Research, Narbonne, France). For the first time, these techniques were used to characterize *ex-situ* microbial communities degrading xenobiotics. The method consists in (i) DNA extraction and purification from sludge sample or enrichment culture with the QIAamp DNA stool Mini Kit (QIAGEN, Hilden, Germany), (ii) amplification of the V3 region of the 16S rDNA genes by using universal primers for eubacteria and archaeobacteria (e.g. primers of Table 1), (iii) analysis of the PCR products by Single Strand Conformation Polymorphism (e.g. on ABI310 Genetic Analyser (Applied Biosystems)), (iv) cloning of the total 16S rDNA in *E.coli* DNA vector (TOPO TA Cloning Kit – Invitrogen), (v) analysis of the single 16S rDNA PCR products by SSCP and identification of the peaks, (vi) sequencing of selected 16S rDNA fragments, (vii) analysis of the results by matching closest sequences in NCBI database (<http://www.ncbi.nlm.nih.gov/>) and building a phylogenetic tree using neighbour joining algorithms (ARB software).

Table 1: Primer sequences used for PCR amplification of, respectively, Archaeobacteria or Eubacterial total 16S SSU rDNA (w2-w17 or w2-w18), V3 region within 16S SSU rDNA before SSCP (w34-w36 or w34-w49) and before cloning (w31-w36 or w31-w49). (6-FAM= 6-carboxyfluorescein, terminal DNA fluorescent label)

Internal Name	Target <i>E.coli</i> posi-		Sequence (5'to 3')
	tion (F-Forward, reverse)	R-	

W02	universal R1492	GNT ACC TTG TTA CGA CTT
W17	Archae F3	ATT CYG GTT GAT CCY GSC RG
W18	Eubacteria F9	GAG TTT GAT CMT GGC TCA G
W31	Universal R500	TTA CCG CGG CTG CTG GCA G
W34	Universal R500 SSCP	6-FAM- TTA CCG CGG CTG CTG GCA G
W36	Archae F333	TCC AGG CCC TAC GGG G
W49	Eubacteria F330	ACG GTC CAG ACT CCT ACG GG

The global strategy used for the characterization of the microbial community by SSCP is described in Fig. 12. In this project, SSCP analyses were periodically performed to investigate dynamics of the microbial populations during the enrichment procedure.

- 5 Only samples corresponding to final enrichment cultures were fully characterized, i.e. cloning and sequencing for microbial identification.

(i) Sampling and total genomic DNA extraction from sludge sample and enrichment cultures. Two-millilitre samples were periodically sampled from bioreactor outlet (CSTR).

- 10 The samples were centrifuged at 6000 g (10 min., 4°C). The pellets were then resuspended in 2 ml of 4 M guanidine thiocyanate-TrisHCl 0.1 M at pH 7.5 and 600 µl of 10% N-Lauroyl-Sarcosine, and stored at -20°C before processing. The extraction and the purification of bacterial genomic DNA were performed with QIAamp DNA stool Mini Kit (Quiagen, Hilden, Germany). The products of extraction were identified by electro-
- 15 phoresis on a 0.7 % agarose gel. DNA bands were detected with 5.10-5 % (w/v) ethidium bromide staining and visualized by UV light.

(ii) Amplification of the V3 region of SSU rDNA genes. First, the total SSU rDNA was amplified by PCR with couples of primers (w02-w17 for the archaeobacteria and w02-w18 for the eubacteria). Primer sequences are presented in Table 1. Each reaction tube contained 0.2 µg of each primer, 1.0 µl of purified DNA, 5µl Taq reaction buffer with 25mM MgCl₂, 0.8 mM dNTP and 1.25 U of Taq polymerase (Stratagene, USA), adjusted to a total volume of 50 µl. The specific V3 region of SSU rDNA was then amplified from the pre-amplified rDNA product with specific primers for conserved domains

20 (w31-w36 for the archaeobacteria and w31-w49 for the eubacteria). Each reaction tube contained 0.13 µg of each primer, 1.0 µl of pre-amplified DNA, 5µl Pfu reaction buffer with 25mM MgCl₂, 0.8 mM dNTP and 1.25 U of Pfu polymerase (Stratagene, USA), adjusted to a total volume of 50 µl. For amplification of the V3 region of the cloned

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fragments, each colony grown overnight was taken and placed in a 0.2 ml microtube PCR (Eppendorf, USA) containing 20 µl of water. All the reactants were then added as described above, and placed in a thermocycler (9600 Perkin-Elmer) at 94°C. For all PCR, three-stage cycles were performed 30 times : 94°C for 30 sec, 51°C for 30 sec, 72°C for 30 sec and a final stage 72°C for 10 min. The amplification was checked by loading 10% (v/v) of the PCR product on a 2.0% agarose gel. The bands were detected with 5.10⁻⁵ % (w/v) ethidium bromide staining and visualized by UV light. The expected size of the band was around 200 bp lengths, and was checked using a 150 bp DNA ladder (Promega, Wis., USA).

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(iv) Cloning of V3 regions of total 16S rDNA PCR products. DNA cloning of the amplified V3 region of SSU rDNA fragments from purified total genomic DNA samples was carried out with the use of a TOPO TA Cloning Kit (Invitrogen). A PCR on the preamplified total 16S rDNA was performed to amplify selectively the V3 region. Couples of primers (w31-w36 for the archaeobacteria and w31-w49 for the eubacteria) were used. PCR amplification was performed following a three-stage cycles 25 times : 94°C for 1 min, 51°C for 1 min, 72°C for 1 min and a final stage 72°C for 10 min. The PCR product was then utilized for cloning according to TOPO TA Cloning Kit recommendations (Invitrogen Product Manual).

20

(iii) and (v) Single Strand Conformation Polymorphism Analysis. SSCP analysis relies on the fact that a single base modification can entirely change the conformation of single strand DNA molecule leading to a different electrophoretic mobility in a non-denaturing gel. Fluorescent dye-labelled PCR primer was used to detect specifically one strand of each DNA fragment by laser detection, and the optimal band separation was reached in an automated DNA sequencer. A size standard was used to compare accurately the patterns from different samples by utilizing a different fluorophore (Genescan-400 Rox; Applied Biosystems) and after computing correction (Genescan software, Applied Biosystems). The SSCP electrophoresis was performed as follows: One µl of diluted PCR product was added to 18.75 µl of loading HIDI formamide (Applied Biosystems). The sample was then denatured for 5 min at 94°C and placed directly on ice for 10 min. SSCP was performed using ABI310 Genetic Analyser (Applied Biosystems), equipped with a capillary column (47 cm x 50 µm) filled with a mixture of Genescan polymer (Applied Biosystems) 5.6 %, glycerol 10 % and TBE 1x (Sigma). Electrophoresis was carried out at 12 kV and 32°C for 30 min per sample. Data processing was performed with the ABI Prism 310 Collection Software (Applied Biosys-

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tems). The second order least square size calling method was used to analyse each sample and normalize mobility from different runs (Genescan Analysis 2.0.2 Software, Applied Biosystems).

- (vi) Sequencing of SSU rDNA genes from clones. The clones were further analysed for microbial identification of the SSCP peaks (see Appendix A2). Plasmid inserts were amplified by PCR with pGEMt primers T7 and P13. PCR products were further sequenced using the dideoxy-chain-termination method, and the ABI model 373A sequencer apparatus (Applied Biosystems). Plasmid DNA was sequenced with the dye-terminator cycle-sequencing reaction kit with AmpliTaq DNA polymerase FS kit buffer (Perkin-Elmer) and the pGEMt primers T7 and P13. A partial sequence of 160 – 200 bp, corresponding to the V3 region was determined for each clone.

- Phylogenetic analysis. An equal portion of SSU rDNA (E.coli position 326 to 450 / ~200pb) was used for the sequence analysis. Sequences from clones were submitted to Genbank for preliminary analysis using the Blast program of the Ribosomal Database Project to identify putative close phylogenetic relatives. Sequences were aligned to their nearest neighbour with the automated alignment tool of the ARB program package (<http://www.arb-home.de/>). The sequences were tested for chimera structure using the RDP analysis service Check Chimera and during manual inspection of alignment. The phylogenetic tree showing relationship of 16S rDNA archaeal clones sequences was generated by neighbour-joining analysis and corrected with a filter that included only close sequences. One thousand bootstrap analyses were performed and percentages greater than 50% were reported on the phylogenetic trees.

- Phylogenetic identification of the isolates. The species identification of the xenobiotic-degrading isolates was performed by sequencing the full 16S rDNA fragment (primers w2-w18 for Eubacteria / ~1500pb). Each colony grew 2 to 4 days on Petri plates, and was placed in a 0.2 ml PCR microtube (Eppendorf, USA) containing 20 µl of water. The sequencing step was carried out as described above. Sequences were analysed by using the ARB software package and BLAST software from NCBI database. Related microorganisms were identified and a phylogenetic tree showing relationship of 16S rDNA archaeal clones sequences was generated by neighbour-joining analysis. One thousand bootstrap analyses were carried out, and percentages greater than 50% were reported on the phylogenetic trees

Analytical methods

Since the enrichment cultures were of low solid contents and, highly concentrated in xenobiotics, a new analytical method for xenobiotic analysis has been developed and
5 used (see below).

Xenobiotic analysis (new method): Because of the low homogeneity of the xenobiotics in water, the reactors were well mixed before taking sample (5 mL). An aliquot of 1mL of sample was transferred into a 12mL Pyrex tube containing 7mL of water pH12 and
10 2mL of extraction solvent (Pentane: Diethyl Ether 15:85 v:v containing 5mg/L of Fluoranthene-d10). The tube was capped with a Teflon lined stopper and shaken at 170rpm for 24 hours in darkness on a tube rotator (Struers, Gerhardt, Germany). The sample was then centrifuged at 1750rpm for 10 minutes (Rotanta46, Tuttlingen, Germany). Thereafter, 0.5mL of supernatant was added to 0.5mL of the internal standard
15 solution (Pentane: Diethyl Ether 15:85 v:v containing 1mg/L of Phenanthrene-d10). The mixture was finally transferred to a 2mL GC vial for analysis.

The PAE analyses were performed by Gas-Chromatography coupled to mass spectrometer (GC, Agilent 6890N; and MS, Agilent 5973). All the samples were injected
20 split-less into the GC by an automatic sampler. The GC was equipped with a HP-5MS column. The injector and auxiliary temperature was respectively of 250°C and 270°C. Helium was used as carrier gas with a constant flow of 0.8ml/min. The oven temperature was initially set at 60°C for 1 minute. Then an increasing temperature rate of 12°C/min. was applied up to 310°C which was kept for 10 minutes. Then, the temperature
25 was increased by 20°C/min. to 340°C kept for 2 min. Detection by MS was run in scan-mode within the ratio 35 and 330 m/z from 5 to 20 minutes.

Total and Volatile Solid contents: An aliquot of 5mL of sample was dried at 105°C for 24 hours and weighted (TS). The sample was then dried at 550°C for 2 hours and
30 weighted (mineral contents). The VS contents were calculated from these two experimental values.

Biogas methane analysis: After equalization at room temperature, 0.2 mL of reactor headspace was injected into a Gas-Chromatography column (Supelco 2-5320-U Vol-
35 co™-30m x 0.53mm x 3mm). The column temperature was set at 30°C, while injector

and detector temperatures were of 130°C (Shimadzu GC-14A). Helium carrier gas pressure was of 1 kg/cm².

Volatile Fatty Acid (VFAs) Analysis by GC-FID: Acetate, propionate, butyrate, valerate

- 5 and isovalerate VFAs were analysed by Gas Chromatography – Flame Ionization Detector (HP5890 series II, Hewlett-Packard – HPFFAP column 30m x 0.53mm x 1µm). First, 1.5mL of sample was centrifuged in 2mL Eppendorf tube (12000 rpm, 2min.). 50µL of a solution of 25% orthophosphoric acid was then added in 950µL of supernatant and transferred in GC vial for analysis. The following GC parameters were used
- 10 : Hydrogen pressure, 100kPa; Nitrogen pressure, 276kPa; initial oven temperature, 70°C; increasing temperature rate, 10°C/min.; final oven temperature, 190°C; injector temperature, 150°C; detector temperature, 200°C.

Example 1.

15

Comparison of PAE enrichment techniques

Emphasis of the PAE-degrading ability of anaerobic ecosystems in batch reactors.

- 20 Several tests were performed in batch reactors in order to demonstrate PAE biodegradation under anaerobic conditions. Two types of anaerobic ecosystems were used: sewage sludge - SS – taken from a Danish WWTP and a household-waste-digesting sludge - HW -. Two activators of the bacterial adhesion were also added in SS sludge: bentonite - BEN - and activated carbon -AC – (both 10 g/L). The results are presented
- 25 in Fig. 1 (initial PAE concentration at 10 mg/L). The controls correspond to sterile reactors indicating PAE abiotic losses.

- Significant biodegradation of PAE in SS sewage sludge was observed with around 70 % of losses due to biological activity. With the HW sludge, only 12% of biological degradation was observed. It was therefore concluded that the SS sludge exhibits a specific and highly efficient biological activity on PAE, whereas the PAE removal rates measured with household waste digested sludge likely result from a residual non-specific biological activity.
- 30

- In addition, both activated carbon and bentonite-added reactors showed high removal efficiencies (75% to 90%). However, similar ranges of removal were measured in controls showing clearly the effect of the strong adsorption of the PAE on activated carbon
- 35

and on bentonite during the process. It was therefore concluded that removal is mainly due to abiotic losses in presence of bentonite or activated carbon.

The SS sewage sludge was finally chosen for further use as initial inoculum during the enrichment procedures, because of the high specificity and high efficiency of its biological activity.

Comparison of fed batch and continuous enrichment cultures.

- Two enrichment procedures, corresponding either to a series of successive fed-batch enrichments or to a continuous enrichment in continuous stirred tank reactor (CSTR), were tested under anaerobic conditions (PAE at 10 mg/L for both). In order to consider the specific PAE-degrading ability of the different ecosystems, the results are expressed as specific removal rates in mg of PAE degraded per day and per gram of volatile solids (biomass). The biodegradability tests were performed in batch reactors by assessing PAE degradation over enrichment time (initial concentration at 10 mg/L DBP). Each series of batch reactors was periodically carried out every 20 days of enrichment. This period corresponds to a complete filling out of one fed-batch reactor, and to the hydraulic retention time of the CSTR reactor.
- The results are presented in Fig. 2 (control reactors are not represented here because abiotic losses can be considered as insignificant). Clearly, the CSTR enrichment culture exhibited a significant increase of the biological removal rates after three to four hydraulic retention times of enrichment (80 days), likely due to a strong washout of the slow growing microorganisms. The fed-batch enrichment procedure was almost similar to CSTR for the first 40 days and seems then to be likely slowed down by the presence of "non-useful" competitive bacteria growing on yeast extract or cell death material. Enrichment of xenobiotic degraders in CSTR is therefore the most efficient procedure to obtain rapidly highly enriched cultures.

PAE degradation range of the enrichment culture.

- The PAE degradation range of the enrichment culture was here tested on two PAE compounds: the Di-Butyl-Phthalate (DBP) and the Di-Ethyl-Hexyl-Phthalate (DEHP). The microbial PAE-degrading activities were determined in batch reactors with an initial concentration of 10 mg/L each, and activities are expressed as specific removal rates in mg PAE degraded per day and per gram of volatile solids (biomass). The enriched and non-enriched cultures correspond to continuous reactors fed, respectively, with and

without PAE at 10 mg/L (80 days of enrichment). The Fig. 3a and 3b show the DBP biodegradation rates of the enriched or non-enriched cultures, and in abiotic controls. Since the abiotic losses are insignificant, it is here demonstrated that the PAE degradation ability was greatly enhanced during the enrichment procedure. In conclusion, such enrichment culture exhibits an interesting range for PAE degradation, especially for the compounds concerned by the current Danish and future EU regulations, the DBP and the DEHP.

Batch tests on different carbon sources and increasing PAE concentrations.

10 The impact of different co-substrates and increasing concentrations of PAEs on xenobiotic removal and methanogenic activity was investigated. Several methanogenic substrates were chosen as study models, such as acetate, propionate, butyrate, glucose and H₂/CO₂. The studies were carried out in batch reactors during 10 days, and the initial concentrations of acetate, propionate, butyrate, glucose and H₂/CO₂ were, respectively, of 5mM, 5mM, 5mM, 5g/L and 1 bar. The range of PAE concentrations was of 0, 10, 100 and 250 mg/l of Di-Butyl-Phthalate (DBP). The PAE, Volatile Fatty Acid (VFA) and VS contents were measured every second day. The effect of co-substrates and PAE concentrations on PAE removal, VFA removal and methane production rates was investigated as follows:

- 20 - The PAE removal rates are presented in Fig. 4a and 4b and for, respectively, the enriched and non-enriched cultures. Low inhibition of PAE removal rate was observed in enriched culture for PAE concentrations above 100 mg/L, and for all substrates, except for acetate where inhibition was stronger at lower concentrations. These results suggest that PAE biodegradation under anaerobic conditions is controlled by substrate inhibition. In addition, since no substrate effect was observed except for acetate, this compound can be involved as well in the biodegradation pathway, likely as a by-product which retro-inhibits the degradation activity. This pathway would therefore be subjected to substrate and product inhibitions. This assumption is confirmed with the non-enriched culture where a stronger inhibition occurred at high DBP concentrations and with acetate as substrate.

Hence, the enrichment of the culture in DBP-degrading microorganisms was here highly beneficial for the enhancement of the reaction specificity by reducing the inhibition effects of the substrate (DBP) and the product (acetate).

- 35 - VFA removal rate according to the PAE concentration was also investigated.. The enriched culture showed a strong inhibition of total VFA removal according to the increasing PAE concentrations, for all substrates. The methanogenic bacteria

- seem therefore to be well affected by the presence of PAE in the medium. Moreover, total VFA removal rates in case of acetate as substrate became negative for the highest PAE concentrations. This result confirms that acetate is potentially an intermediate of the DBP pathway because of its accumulation. This result is well confirmed in the non-enriched culture where the acetate higher accumulates due to a stronger inhibition of the non-acclimated methanogenic bacteria. For all substrates, it was shown a stronger inhibition of the VFA degradation in the non-enriched than in the enriched culture. In conclusion, the acclimation procedure in CSTR was highly beneficial for the PAE degradation process by selecting hardly the PAE-degrading bacteria as well as the specific syntrophic partners growing on the inhibitory products of the PAE pathway, such as acetate.
- The last part of the study was focused on methane production. An inhibitory effect was also observed. Methane production rates were higher for the enriched than for the non-enriched sludge, confirming the high selectivity of the enrichment procedure. As a consequence of the strong inhibition of PAE on total VFA removal and on PAE degradation rates, an optimum for 50-100 mg/L of PAE was found for the enriched cultures.

Example 2.

Bioaugmentation tests with PAE enrichment cultures

- Preliminary bioaugmentation tests were performed in batch reactors in order to assess the benefit of adding a known amount of the PAE enrichment culture into an anaerobic ecosystem presenting low PAE degradation ability. In this experiment, increasing amounts of enriched culture obtained as in example 1 were added to the anaerobic household-waste digested sludge, from 0 % to 50 % of the total volume (100 ml). The PAE degrading activities were determined with an initial concentration of 10 mg/L of PAE. The results are presented in Fig. 5. The activities are expressed as specific removal rates in $\text{mg}_{\text{PAE degraded}} \text{ per day and per gram of biomass (VS)}$. The control reactor corresponds to a chemically sterilized reactor showing that the abiotic losses were non significant during the process. Without any addition of enrichment culture (0%), the HW sludge exhibited a low removal rate ($0.2 \text{ mg}_{\text{PAE deg. g}_{\text{VS}}^{-1} \cdot \text{d}^{-1}}$). The addition from 5 to 10% v/v of enriched culture enhanced significantly the degradation rates. An optimal ratio was found with addition of 25% v/v of enrichment culture grown in batch reactor for 1 week in presence of DBP at 10 mg/L which showed a great increase (4,2 times) of

PAE removal rate. The approximate number of bacteria per ml in the added cultures corresponds to around 10^2 - 10^9 bacteria/ml, more particularly from 10^4 - 10^6 bacteria/ml. No beneficial gain was measured by addition of 50% of enriched cultures likely because of the strong competition between microorganisms and the low concentration of PAE which could not provide enough energy sources to the added community.

Physiological activity of the enriched and non-enriched cultures

The anaerobic cultures were enriched in continuous perfectly mixed reactors (CSTR) working under mesophilic anaerobic conditions (35°C) for more than four hydraulic retention times (total 100 days). The reactors were fed with a mixture of synthetic BA medium, supplemented with Yeast Extract (2 g/L) and PAE at 0 mg/L, 10mg/L or 200 mg/L (Di-Butyl-Phthalic Acid Ester). Additionally, two control reactors amended with 10 mg/L and 200 mg/L of PAE were implemented in order to assess PAE abiotic losses due to the process. The control reactors were chemically sterilized by addition of 2% Formaldehyde and 1.4% Sodium Azide. PAE removal values were calculated from a reactor mass balance assessment at steady state, and correspond to average losses. Results are presented in Table 2. Average biogas production corresponds to the volume of biogas produced per week, and is highly representative of the methanogenic activity of anaerobic microbial ecosystems. The results showed a strong inverse correlation between decreasing biogas production rate and the related increasing PAE concentrations in inlet. This suggested an inhibitory effect of the PAE. Since biogas production was not significant in control reactors, it can be concluded that they were well sterilized, and that they can accurately inform on abiotic losses of PAE. As presented in Table 2, PAE abiotic losses reached 25% at 10 mg/L and 30% at 200 mg/l. More than 90% to 99% of PAE were degraded in biological reactors. Therefore, PAE biodegradation under anaerobic conditions was here highly efficient (>60%). Actually, the final PAE concentration was around 2 mg/l in both cases. Below this concentration, PAE bioavailability could be the main limiting factor for PAE biodegradation. Since CSTR reactors presented high PAE removal efficiencies during the enrichment procedure, they were used to characterize dynamically the microbial community degrading PAE under anaerobic conditions.

Table 2: Phthalic Acid Ester (PAE) removal and impact on methanogenic activity

PAE concentration (Inlet) (mg/l)	PAE removal at steady state (%)	Average biogas produc- tion rate [70% CH ₄] (ml/week)
0	-	41.7 +/- 1.5
10	89.3 +/- 1.3	38.0 +/- 3.5
10 (sterile control)	24.3 +/- 7.9	0
200	99.3 +/- 0.3	13.7 +/- 3.2
200 (sterile control)	31.4 +/- 11.4	0

Characterization of Microbial community by SSCP.

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The *ex-situ* characterization of the archaeal and eubacterial populations by SSCP was carried out on samples taken from the blank (0mg/L) and the PAE-supplemented (10mg/L – 200mg/L) reactors, at steady state. The SSCP chromatograms are presented in Fig. 13. Clearly, a shift of the archaeal community was observed between the blank and the highest concentration (200 mg/L). At 10 mg/L, an intermediate case associating both profiles was observed likely because of a lower selection pressure. Therefore, the addition of PAE not only inhibited specifically the initial archaeobacteria population resulting in the reduction of the methanogenic activity, but selected also new resistant methanogenic bacteria suggesting the establishment of a new symbiotic consortium in the enriched culture. Indeed, several specific eubacteria were selected as well during the process. The eubacterial population presented also graduated results according to PAE concentrations with an intermediate case at 10 mg/L (see chromatograms in Fig. 13). Three bacteria (*bact1*, *bact2* and *bact3*) were identified as being potentially selected by the increasing concentrations of PAE, with *bact1* as the main dominant species (200mg/L PAE). Since *bact2* and *bact3* were not found in the 10 mg/L reactor, these results suggest that *bact1* would effectively grow on PAE and the other two bacteria *bact2* and *bact3* would participate to the PAE pathway as opportunistic bacteria by growing on by-products. A dynamic characterization of the enrichment cultures was also performed in order to confirm our previous assumption. Dynamics of the archaeal and eubacterial microbial population were assessed by SSCP over experimentation time, as follows:

Eubacteria. In order to simplify the representation of the SSCP chromatograms according to experimental time, the relative abundance of each SSCP peak corresponding to bacterial species dynamics over time are represented in Fig. 14 to 16. They were calculated and formatted by internal software analysis especially developed for this study. At start of the experiment, several dominant species were identified such as peak n°14 (18.7%), and peaks n°15, n°17, n°38 from 10.4 % to 11.5% of total population. In this inoculum, the peak abundance was completely distributed along the chromatograms. After enrichment, a shift of the eubacterial population occurred in the blank reactor with a progressive disappearance of the dominant peak n°14, and with the emergence of new dominant species n°36 (38%) and n°38 (11%). These species were therefore suspected to grow on the sole carbon sources available in the inlet, i.e. on Yeast Extract or on PAE dissolution solvent (added without PAE in blank reactor). By constant addition of 10mg/L of PAE, similar results were observed for peaks n°14, n°36 and n°38, but with the emergence of a new dominant eubacteria representing around 33% of the population at the end of the enrichment procedure (peak n°9 corresponding to bact1 in Fig. 14). This shift of the dominant species occurred approximately after 11 weeks of enrichment (Fig. 15). By increasing the inlet PAE concentration to 200 mg/L, selection of peak n°9 was speed up with a change of the dominant population after only 5 weeks of enrichment (Fig. 16). Therefore, higher PAE concentrations were beneficial to enhance the selection of the PAE-degrading bacteria. As well, two other sub-dominant species corresponding to the peaks n°28 and n°40, appeared at the end of the experiment with, respectively, 11% and 21% of the population (bact2 and bact3 in the chromatogram in Fig. 13). As previously suggested, these sub-dominant species may be opportunistic bacteria growing on end-products from the PAE pathway, because of their lower abundance in all PAE-supplemented reactors. Since a single peak mostly corresponds to one single species according to the SSCP specificities, the cloning and sequencing steps provided some identification information on these microorganisms. Results are presented in the eubacterial phylogenetic tree (see Fig. 20). Bact2 (peak n°28 – new uncultured *Bacteroides* sp) and bact3 (peak n°40) corresponds to the same bacteria (100 % similarity). Actually, peak n°28 may result from the isomerisation of the 16S rDNA secondary structure. In addition, the couple bact2/bact3 is located in the same phylogenetic order than bacteria found in the blank (bact4, peak n°36 *Bacteroides* sp. AY144265 at 6,6% divergence) within the *Porphyromonadaceae* family and the *Bacteroides* class gathering bacteria widespread in nature (9.5% divergence between bact2/bact3 and blank bact4 suggesting that they belong to different genera).

This result confirms that these microorganisms were likely opportunistic bacteria growing on general end-products from the PAE pathway because of the non specificity of the phylogenetic group. Another dominant blank bacterium was found to be a *Spirochaeta Buddy* sp. (0.49 % divergence), and was used as control during the FISH procedure. In contrast, the dominant species in the PAE enrichment cultures (bact1 - peak n°9) was identified as *Soehngenia saccharolytica* AJ582209 (100 % similarity) already described in the literature as presenting anaerobic aromatic benzaldehyde-degrading activity. Since the molecular structure of PAE is very close, it was here confirmed that this eubacteria could be directly involved in anaerobic PAE-degradation. To date, this bacterium has not been isolated in our laboratory. Nonetheless, specific FISH probes were designed and tested for monitoring the abundance of the degrading consortium and describe their spatial configuration. It appears that the dominant specie (bact1) was mainly found in flocs in the enriched cultures while bact2/3 group is spread in the medium without any specific spatial arrangement. This may suggest that the dominant specie of the enriched culture grows essentially where hydrophobic compounds such as PAEs are trapped, i.e. into the biofilm matrix. Moreover, as shown in the next section, this kind of spatial arrangement favoured symbiotic relationships with methanogens. Relative abundance was also confirmed by the dominance of bact1 in the enriched culture where no or few blank bacteria were found.

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Archaeobacteria. Similar dynamic characterization was performed for the archaeal community. None difference of archaeal population was observed in the blank reactor over experimental time (Fig. 17 arch3 - peak n°5 – around 80% of the population). Since no inhibitory effect or an increase of a specific carbon source occurred in blank reactor, a specific archaeal population did not have to be selected on yeast extract according to their physiological properties of using classical end-products of the hydrolysis step (mainly H₂/CO₂, volatile fatty acids,...). However, by addition of 10mg/L of PAE, new dominant archaeal bacteria were selected after 12 weeks of enrichment (see Fig. 18 – arch4 – peak n°9 – 78% at the end). By addition of 200mg/L of PAE, the selection of the new dominant archaeobacterium occurred after only 4 weeks of enrichment (see Fig. 19 – arch4 – peak n°9 – 78% at the end). Therefore, the increase of PAE concentration in reactor inlet inhibited progressively the starting population by selecting resistant archaeal species. Furthermore, the shifts of eubacterial and archaeal populations were almost synchronous, suggesting the establishment of new physiological symbiotic relationships between the dominant eubacteria and archaeobacteria throughout the PAE degradation pathway. In addition, cloning and sequencing steps

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were carried out as well as for the study on eubacteria. Results are presented in the archaeobacteria phylogenetic tree (see Fig. 21). Species arch3 (peak n°5 in blank) and arch4 (peak n°9 in enrichment culture) are phylogenetically very similar and both are belonging to the genus *Methanosaeta* (3.9 % divergence between the two microorgan-

5 isms suggesting that arch3 and arch4 belong to the same genus but do not represent the same species). Therefore, the genus level are not low enough to figure out the selection of arch4 instead of arch3, which may results from species properties, such as specific adhesion marker or high hydrophobic membrane properties. This latter could be particularly true in case of symbiotic relationships with eubacteria in PAE pathway

10 because of the very low bioavailability of these compounds. Indeed, as PAE are highly hydrophobic and lowly soluble in water, the hydrophobic properties of the symbiotic bacterial consortium could be a major key point for the selection of involved microorganisms. In addition, a FISH probe was designed to state the spatial arrangement between the archaeal and eubacterial species (see Deliverable 9.1). Because of their hy-

15 drophobic properties, *Methanosaeta* sp. are mostly found in flocs, that favours the contact between hydrophobic compounds, the degrading bacteria and the symbiotic methanogens. Here the results showed clearly the involvement of a symbiotic consortium in flocs likely involved in the PAE degradation. Such selection may occur between very specific microorganisms although the methanogens selection was mainly based

20 on their morphological/phylogenetic properties rather than physiological properties (mainly acetate degrader).

Example 3

25 Characterization of four anaerobic PAE-degrading isolates

Isolation of DBP degraders

Starting from complex enrichment cultures obtained as in example 1, the specific technique to obtain DBP degrading isolates was based on successive selection of bacteria

30 with an anaerobic synthetic medium, and DBP as sole carbon source. The enrichment cultures were first enriched in fed-batch reactors with semi-continuous addition of synthetic medium amended with DBP as carbon source.

35 The first isolation step was carried out by cultivation of pure to 10^{-2} dilutions of the above highly enriched culture on synthetic agar medium with DBP as sole carbon

source (10 mg/L). Four cultivable isolates from this plate were selected as clearly forming independent and distinct colonies: Isolates A, B, C and D. Each isolate was then cultivated separately on new agar plates with a range of DBP concentration from 10 to 500 mg/L in a synthetic medium with added yeast extract (2 g/L). Their ability to growth
5 only on yeast extract or only on DBP was also tested (see table 3)

Isolation Procedure in detail. Fed batch reactors (no effluent) were used as first step in isolating degrading microorganisms from sludge. The reactors were set up with 20 vol % of 16 enrichment culture from the CSTR and 80 vol % of BA-Medium in clean and
10 heat treated 100 mL bottles with rubber stoppers and aluminum ring caps. Two separate series of fed batch reactors were carried out. One containing 0.5 g L⁻¹ yeast extract, and the other one with no yeast extract using PAE as the sole carbon source. The bottles containing the appropriate amount of BA-Medium were flushed with a gas mixture of N₂:CO₂ (80:20 v:v) and stored at 35°C in the dark on a shaker. The required
15 amount of enrichment culture was then added to the reactors directly after sampling from the CSTRs. Afterwards, the reactors were fed with PAE solutions for 8 days to reach a final concentration of 100 mg L⁻¹ assuming 0 mg L⁻¹ at start. This procedure was repeated twice using 20 vol % of the previous fed batch reactor instead of enrichment cultures.

20 Then, samples of 1 mL were taken from each of the fed batch reactors and spread on agar plates using a Drigalski spatula. Plates were prepared by adding 15 g L⁻¹ pure bacterial agar and 0.5 g L⁻¹ yeast extract for one set of the BA-Medium before autoclaving. After autoclaving 100 mg L⁻¹ of PAE, vitamins and Na₂S were added to the still warm medium under sterile conditions. The medium was mixed, and then approxi-
25 mately 20 mL were poured into empty Petri dishes. The plates were kept under a sterile fumehood until the agar became solid. The plates were subsequently stored in an anaerobic jar with an oxygen trap (ANAEROCULT A, Merck, Darmstadt, Germany) for a minimum of 24 hours prior to use.

Finally, the isolates were tested for degradation potential in 10 mL Pyrex tubes with teflon screw caps. Tubes were thoroughly washed and held at 220°C for a minimum of 12
30 hours to remove any trace organic residue prior to use. Tubes were then closed and autoclaved for 30 min at 121°C (Syste V-65 Autoclave, Brønby, Denmark). After autoclaving, 10 mL of BA-Medium was prepared as described above with 100 mg/L PAE, and 0.5 g L⁻¹ or 0 g/L yeast extract respectively was added to the tubes under sterile
35 conditions. Colonies were then added to the liquid medium using an inoculation loop. Tubes without bacteria, but otherwise prepared identically, were used as controls. The

headspace of the tubes was flushed with a gas mixture of N₂:CO₂ (80:20 v:v) through a sterile filter (0.20 µm, Sartorius) and closed with the teflon screw cap immediately. Tubes were incubated at 35 °C in the dark on a shaker for 14 days.

5 Batch Experiments with Isolates.

For more detailed kinetic studies on selected isolate cultures, batch experiments were performed in 100 mL bottles with rubber stoppers and aluminum ring caps. Isolated cultures were taken from agar plates with an inoculation loop into 12 mL flat bottom tubes with teflon screw caps for pre-culturing. Tubes were washed and put at 220°C for a minimum of 12 hours and were then closed and autoclaved for 30 min at 121°C prior to use. Tubes contained 10 mL of BA-Medium with 100 mg L⁻¹ of PAE and 0.5 g L⁻¹ or 0 g L⁻¹ yeast extract, respectively. After adding the colonies, the pre-culture tubes were incubated for 6 days at 35 °C in the dark on a shaker. Microbial growth was followed by turbidity, measuring optical density (OD) at 605nm 2-3 times a day using a photometer (Spectroquant Nova 60, Merck, Germany). The batch experiments were then performed using 95 mL of the respective BA-Medium with a concentration of 100 mg L⁻¹ PAE and 5 mL of the pre-culture. Bottles were closed with a rubber stopper after adding 95 mL of BA-Medium and the headspace was flushed with a gas mixture of N₂:CO₂ (80:20 v:v). An amount of 5 mL of the pre-culture was added to the reactor with a 5 mL plastic syringe and a sterile needle. Control reactors were operated with pure BA-Medium. The reactors were kept at 35°C in the dark on a shaker and were sampled for a period of 20 days.

All isolates showed an ability to grow on plates amended with a range of PAE, suggesting a low specificity of the first step of degradation (Table 4). Isolate D showed however an ability to grow on agar as sole carbon source, but not the other isolates.

Additionally, a range of different carbon sources were tested to fully characterize the physiology of isolates A, B, C and D under aerobic and anaerobic conditions (all isolates are anaerobic facultative). The growth on carbon sources were tested on microplates, and the results are presented in Table 6.

Table 3: Growth characteristics of four isolates according to the addition of increasing PAE (DBP) concentrations in the culture BA-medium (“-”, “+”, “++”, “+++” respectively indicate no growth, weak, normal, good and very good growth). (YE = Yeast Extract). A: *Exiguobacterium* sp. strain DBPA (DSM 17351) , B: *Exiguobacterium* sp. strain

DBPB (DSM 17352), C: *Bacillus* sp. strain DBPC (DSM 17353), D: *Bacillus* sp. DBPD (DSM 17354).

isolate	DBP concentration (YE=2g/l)										DBP(100mg/l)		No DBP	
	10		50		100		250		500		No YE		YE (2g/l)	
A	++	+++	++	+++	+++	+++	+++	+++	++	++	-	-	++	++
B	++	+++	++	+++	+++	+++	+++	+++	++	++	-	-	++	++
C	++	+++	++	+++	+++	+++	+++	+++	++	++	+	+	-	-
D	++	+++	++	+++	+++	+++	+++	+++	++	++	+	+	+	+

- 5 Table 4: Growth characteristics of the four isolates on different carbon sources at 100 mg/L (“-”, “+”, “++”, “+++” respectively indicate no growth, weak, normal, good and very good growth). (YE = Yeast Extract)

isolate	DMP	DBP	DEP	BBP	DEHP	Phthalic acid	Benzoate	Only agar
A	++	++	++	++	++	++	++	-
B	++	++	++	++	++	++	++	-
C	++	+++	+++	+++	+++	+++	+++	-
D	++	+++	+++	+++	+++	+++	+++	+

Morphological characterization of the isolates:

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The first isolation step of the four isolates showed the morphology of the isolate colonies. The colony colour is common for all isolates and is white and translucent. However, two kinds of colony morphology can be distinguished: isolates A and B (A: *Exiguobacterium* sp. strain DBPA (DSM 17351) , B: *Exiguobacterium* sp. strain DBPB

- 15 (DSM 17352)) form round and individual smooth colonies, whereas C and D (C: *Bacillus* sp. strain DBPC (DSM 17353), D: *Bacillus* sp. DBPD (DSM 17354)) isolates form non individual wavy and irregular filamentous colonies, suggesting the presence of motile bacteria. This form of colony is enhanced if the concentration of DBP is low (see Table 5). The cellular morphology of the isolates has also been characterized. Isolate A
- 20 is a rod shaped non motile bacterium and isolate B is a rod-coccus morphology non motile bacterium. Isolate C exhibits different morphologies whether the isolate grows on liquid or solid medium. In liquid culture, isolate C is a shaped rod highly motile and spore-forming bacteria while in solid medium, isolate C cells form a highly filamentous

colony (in chains). Similarly, isolate D is a single morphology rod shaped highly motile bacteria in liquid culture, which aggregates on solid medium, as previously described for the isolate C. Morphologies of isolate A and B as well as C and D are highly similar. In addition, the characterization of the isolate growth according to the DBP concentration is presented in Table 3. Optimal growth was reached at 100 mg/l and 250 mg/l of DBP, indicating probable growth inhibition at higher concentrations. Yeast extract was added in the minimum synthetic medium in order to provide essential organic elements such as vitamins or cofactors, but it could also be considered as a carbon source. Isolates A and B were able to grow easily on yeast extract whereas growth of isolate D was weak and growth of isolate C was not observed. In all cases, yeast extract addition was necessary for an optimal growth of the isolates on DBP (possibly to fulfil requirements for specific cofactors and vitamins).

Table 5: Filamentous shape of colony D. DBP = Di-butyl phthalate (PAE); YE = Yeast Extract. ("–", "+", "++", "+++" respectively indicate an increasing filamentous shape on solid agar medium)

	DBP concentration (YE=2g/l)										DBP (100mg/l)		No DBP	
	10		50		100		250		500		No YE		YE (2g/l)	
filamentous shape of colony D	+++	+++	++	++	+	+	+	+	-	-	+	+	+	+

20 Physiological characterization of the isolates:

Firstly, the ability of the four isolates to degrade PAE under anaerobic conditions was first tested in batch reactors filled with BA medium supplemented with 30 mg/L of DBP (Di-butyl-phthalic acid ester) and Yeast Extract (2 g/L). PAE removals were determined after two days of cultivation by measuring final PAE concentration. Therefore, four batch reactors were inoculated with the four isolates A,B,C and D, as well as two control reactors (no bacteria), one reactor with isolate D but without Yeast Extract and one blank reactor inoculated with X bacteria growing on Yeast Extract only. Results are presented in Fig.6. As shown in control reactors, the abiotic losses can be considered as insignificant during the experiment (< 5%). Each isolate exhibited different removal

performances from around 50% for isolate A for more than 90% for isolate C. Physiologically, the four isolates presented different PAE degradation properties. Moreover, the addition of yeast extract in the cultivation medium was not necessary for isolate D who grew weakly but effectively on PAE. The specific biological degradation of PAE by the four isolates rather than a single adsorption of the compound into bacterial cells was furthermore confirmed with isolate X, where PAE removal remained less than 20% while the bacteria grew well on yeast extract. Therefore, specific PAE biodegradation suggesting the presence of specific potential was here demonstrated for the four isolates.

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The ability of the isolates to degrade other PAEs was also evaluated. Similar results were observed with DEHP at 50 mg/l (Fig.7). No degradation was observed in the control. Even though they were isolated on DBP, efficient biodegradation of the DEHP was observed for all isolates, showing the ability of these strains to degrade a wide range of PAE.

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Additional tests on agar plates supplemented with DMP, DBP, DEP, DEHP or BBP as sole carbon source showed a growth in all cases. Therefore, the pure strains were able to degrade and metabolize all of the cited PAE compounds.

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Secondly, a range of different carbon sources were tested to fully characterize the physiology of isolates A, B, C and D under aerobic and anaerobic conditions (all isolates are anaerobic facultative). Results are presented in Table 6.

25 **Table 6:** Physiological characterization of four PAE-degrading isolated bacteria

	Carbon sources	Isolates					Carbon sources	Isolates			
		A	B	C	D			A	B	C	D
Aerobic	Pyruvic acid methyl ester	+	+	+	+	Anaerobic	Lactulose	+	+	+	+
	Tween 40	+	+	+	+		Palatinose	+	+	+	+
	Tween 80	+	+	+	+		Glyoxylic acid	+	+	-	-
	□-Cyclodextrin	+	+	+	+		Pyruvic acid	+	+	+	+
	Glycogen	-	-	-	-		L-alanyl-L-Threonine	-	-	-	-
	D-Cellobiose	+	+	+	+		L-valine	+	+	-	-

ATP	aerobic	□-D-Lactose	+	+	+	+		Amygdalin	-	-	-	-
		□-methyl-D-Glucoside	+	+	+	+		D-Galacturonic acid	+	+	+	+
		D-Xylose	+	+	+	+		Maltose	+	+	-	-
		i-Erythritol	+	+	+	+		D-Raffinose	-	-	-	-
		D-Mannitol	-	-	-	-		□-hydroxy Butyric acid	-	-	-	-
		N-acetyl-D-Glucosamine	-	-	-	-		Pyruvic acid methyl ester	-	-	-	-
		D-Glucosaminic acid	-	-	-	-		L-Asparagine	-	-	-	-
		Glucose-1-phosphate	+	+	+	+		L-Valine plus L-Aspartic acid	+	+	-	-
		D,L-□-Glycerol phosphate	-	-	-	-		D-Arabitol	-	-	-	-
		D-Galactonic acid □-lactone	+	+	+	+		Gentiobiose	+	+	+	-
		D-Galacturonic acid	+	+	+	+		Maltotriose	+	+	-	-
		2-hydroxy-Benzoic acid	+	+	+	+		L-Rhamnose	+	+	+	+
		4-hydroxy-Benzoic acid	-	-	-	-		□-hydroxy Butyric acid	-	-	-	-
		□-hydroxy-Butyric acid	-	-	-	-		D-Saccharic acid	-	-	-	-
		Itaconic acid	-	-	-	-		L-Glutamic acid	-	-	-	-
		□-keto-Butyric acid	+	+	+	+		2'-deoxy-Adenosine	-	-	-	-
		D-Malic acid	-	-	-	-		Arbutin	-	-	-	-
		L-Arginine	-	-	-	-		D-Gluconic acid	-	-	-	-
		L-Asparagine	-	-	-	-		D-Mannitol	-	-	-	-
		L-Phenylalanine	-	-	-	-		Salycin	-	-	-	-
		L-Serine	-	-	-	-		Itaconic acid	-	-	-	-
		L-Threonine	-	-	-	-		Succinamic acid	-	-	-	-
		Glycyl-L-Glutamic acid	-	-	-	-		L-Glutamine	-	-	-	-
		Phenylethylamine	+	+	+	+		Inosine	-	-	-	-
		Putrescine	-	-	-	-		D-Cellobiose	-	-	+	-
		Dulcitol	-	-	-	-		D-Glucosaminic acid	-	-	-	-
		Glycerol	+	+	+	-		D-Mannose	+	+	+	-

□-methyl-D-Galactoside	+	+	-	-
Turanose	+	+	+	+
D-Lactic acid methyl ester	-	-	-	-
Alaninamide	-	-	-	-
L-Methionine	-	-	-	-
N-acetyl-D-Galactosamine	-	-	-	-
i-Erythritol	-	-	-	-
D,L-□-Glycerol phosphate	+	+	-	-
□-methyl-D-Galactoside	+	+	-	-
Acetic acid	+	+	-	-
D-Malic acid	-	-	-	-
L-Alanine	-	-	-	-
L-Phenylalanine	-	-	+	-
N-acetyl-D-glucosamine	-	-	-	-
D-Fructose	+	+	+	+
m-Inositol	-	-	-	-
□-methyl-D-Glucoside	-	-	-	-
Formic acid	-	-	-	-
L-Malic acid	-	-	-	-
L-alanyl-L-Glutamine	-	-	-	-
L-Serine	-	-	-	-
N-acetyl-□-D-Mannosamine	-	-	+	-
L-Fucose	+	+	+	+
D-Sorbitol	-	-	-	-
□-keto-Butyric acid	+	+	+	+
Succinic acid	-	-	-	-
Glycyl-L-Aspartic acid	-	-	+	-
Thymidin	+	+	-	-
□-Cyclodextrin	-	-	-	-
□-D-Glucose	+	+	-	-
D-Melezitose	-	-	-	-
Stachyose	-	-	-	-
□-keto-valeric acid	+	+	+	+
Succinic acid mono-methyl ester	-	-	-	-
Glycyl-L-Glutamine	-	-	-	-
Uridine	-	-	-	-
□-Cyclodextrin	-	-	-	-
□-D-Glucose-1-Phosphate	-	-	-	-
D-Melibiose	-	-	+	-
Sucrose	+	+	+	-
D,L-Lactic acid	-	-	-	-
m-Tartaric acid	-	-	-	-
Glycyl-L-Methionine	-	-	-	-
Thymidin-5'-mono-phosphate	+	+	-	-
Dextrin	-	-	-	-
D-Glucose-6-phosphate	+	+	+	+

□-D-Lactose	-	-	-	-	3-methyl-D-Glucose	+	+	+	-
□-methyl-D-Glucoside	+	+	-	-	D-Trehalose	+	+	-	-
Fumaric acid	-	-	-	-	L-Lactic acid	-	-	-	-
Propionic acid	-	-	-	-	Urocanic acid	-	-	-	-
L-alanyl-L-Histidine	-	-	-	-	Glycyl-L-Prolin	-	-	-	-
D-Galactose	+	+	+	+	Uridin-5'-mono-phosphate	-	-	-	-

Phylogenetic affiliation of the isolates:

- 5 In order to identify the phylogeny of the isolates, the full 16S rDNA gene of the four strains was amplified by direct PCR on colony. PCR primers corresponded to universal sequences amplifying any 16S rDNA from eubacteria, from E.coli position n°9 to n°1492. The PCR products were then analysed by migration on agarose gel and revealed by ethidium bromide under UV light. The amplified 16S rDNA fragments of
- 10 about 1500 bp were taken from the gel, purified and sequenced. Total 16S rDNA of each isolate was thereafter compared to the official and exhaustive Genbank/NCBI 16S rDNA database using the NCBI BLAST software. Pairwise evolutionary distances were worked out by the Blast software comparing the corrected Pairwise sequences with those in the database, after sequence alignment within the reference 16S rDNA of
- 15 E.coli. Results are reported in Table 7. Isolates A and B were identified as belonging to the same bacterial species *Exiguobacterium gaetbuli*, with a similar sequence homology of 99.57 %. Moreover, direct comparison of 16S rDNA showed a high similarity between both 16S rDNA demonstrating that A and B are two strains of the same species. In contrast, isolates C and D correspond to distinct species, respectively *B.fusiformis*
- 20 and *B.licheniformis*, belonging to the same *Bacillus* genus (Table 7). This result is confirmed by divergence higher than 3 % between isolate C and D sequences demonstrating the species distinction between the two isolates. Therefore two groups were phylogenetically distinguished: A and B isolates are rod shaped non motile bacteria, while C and D exhibit different colony morphology according to the solid or liquid culture and
- 25 correspond to highly motile bacteria forming filaments on solid plates. Indeed, the phylogeny of the isolates was determined by 16S rDNA sequencing, and phylogenetic affiliation. It appeared that isolates A and B correspond to the same species, but likely not the same strain because of distinct PAE degrading ability. This observation may

also result from the presence of a plasmid carrying the PAE degradation genes. In this case, the same species could exhibit different physiological properties due to the presence or absence of a specific plasmid, as previously reported for aromatic degradation genes (PAH). Such property could be very interesting to transfer the PAE degrading ability over different bacterial species in complex environment, and bioaugmentation of natural ecosystems may succeed more readily than expected by monitoring not only the bacterial strain but also the degradation genes. However, this hypothesis still needs to be investigated and confirmed. In contrast, isolates C and D correspond to distinct *Bacillus* species from the same genus, and in a different *Bacillaceae* family compared isolates A and B. Plasmid transfer could have also occurred in this case. The gene identification and characterization will provide a better understanding of the PAE degradation pathway and could be thereafter used as a functional biomarker of the PAE degrading ability of anaerobic ecosystems. It can also be noticed that these four isolates are traditionally described as aerobic bacteria, which was confirmed by their physiological characterization under aerobic conditions. However, they are belonging to a genetic cluster gathering numerous fermentative bacteria, suggesting their possible ability to degrade PAE in a fermentation process.

Table 7: Related microorganisms found in the literature by comparison of full 16S rDNA from PAE-degrading isolates A,B,C and D with NCBI database. Reported species correspond to the highest sequence homology found in the database and reported here (< 2% divergence)

Strain	Reported specie	Sequence homology (%)
A	<i>Exiguobacterium gaetbuli</i>	99.57
B	<i>Exiguobacterium gaetbuli</i>	99.57
C	<i>Bacillus fusiformis</i>	99.65
D	<i>Bacillus licheniformis</i>	98.16

Table 8: Matrix of divergence (%) between 16S rDNA sequences of PAE-degrading isolates A, B, C and D

	A	B	C	D
A	0	0	12.2	10.8
B	0	0	12.2	10.8
C	12.2	12.2	0	8.8
D	10.8	10.8	8.8	0

Phylogenetic relationships

5 Based on the evolutionary distance values, a phylogenetic tree including the four isolates A, B, C and D was built by neighbour joining method using Felstensteins algorithm in ARB software. The phylogenetic tree is presented in Figure 8. As expected, the three isolates belong to three distinct clusters related to each other by more than 8% divergence, but from the same Bacillaceae family. Related to their xenobiotic degrading
10 activity, isolates A and B are close to *Exiguobacterium* sp. BTAH1 degrading atrazine, as well as to a clone found in benzene-degrading sulphate-reducing consortium. Most of the congeners in A/B cluster were found in extreme environment (psychrophilic, halophilic,...) suggesting an ability to resist wether conditions are not favourable, and even under strict anaerobic conditions despite *Bacillus* genus is well know to gather
15 aerobic and anaerobic facultative (fermenting) bacteria. In addition, the congeners of isolate C cluster are often found in strict anaerobic or anoxic environment and are mostly described to be spore-forming fermentative bacteria. This description corresponds to the physiological properties found for isolate C (spore-forming, anaerobic facultative bacteria). A relatively close and interesting congener, *Bacillus benzo-*
20 *evorans*, was also described as degrading aromatic compounds and phenols under mesophilic conditions. At least, isolate D belongs to the same genetic cluster as *Bacillus licheniformis* presenting cellulolytic activity, and producing biosurfactants. Biosurfactants can be helpful for dissolution of hydrophobic compounds such as PAEs and are therefore a physiological advantage in highly selective environment. Moreover, a
25 related congener *Bacillus* sp.63 was found in soils degrading phenanthrene, an aromatic non substituted compound, suggesting the ability of this cluster to participate to aromatic compound degradation pathways. In conclusion, the isolates A, B, C and D could be likely classified as fermentative anaerobic facultative bacteria belonging to the same *Bacillaceae* family, able to grow under extreme conditions and presenting the
30 ability to degrade aromatic compounds.

In conclusion, four anaerobic PAE degrading isolate candidates were obtained by successive dilution on a synthetic medium amended with PAE as sole carbon source, and were fully characterized, physiologically, phenologically and phylogenetically. Well
35 known, a pack of molecular identification tools have been implemented (see deliverable

9.1). Their ability to degrade PAEs is well extended and it is promising for further bio-remediation assays of contaminated sludge.

PAE degradation performances of the isolates

5

In addition, their ability to degrade PAE under anaerobic conditions was investigated in batch reactors filled with BA medium supplemented with 30 mg/L of DBP (Di-butyl-phthalic acid ester) and Yeast Extract (2 g/L). PAE removals were determined after two days of cultivation by measuring final PAE concentration. Therefore, four batch reac-
10 tors were inoculated with the four isolates A,B,C and D, as well as two control reactors (no bacteria), one reactor with isolate D but without Yeast Extract and one blank reactor inoculated with X bacteria growing on Yeast Extract only. Results are presented in Figure 6. As shown in control reactors, the abiotic losses were considered as non significant during the experiment (< 5%). Each isolate exhibited different removal per-
15 formances from around 50% for isolate A for more than 90% for isolate C. Kinetically, the four isolates presented different PAE degradation properties. The addition of yeast extract in the cultivation medium was not necessary for isolate D who grew weakly but effectively on PAE. The specific biological degradation of PAE by the four isolates rather than a single adsorption of the compound into bacterial cells was furthermore
20 confirmed with isolate X, where PAE removal remained less than 20% while the bacteria grew well on yeast extract. Therefore, specific PAE biodegradation suggesting the presence of enzymatic potential was here demonstrated for the four isolates.

Example 4

25 Degradation of PAE in wastewater with and without active biomass (up to 3 g/l), and with or without addition of bacteria according to the invention
Pre treated wastewater from Lundtofte WWTP (after primary settling) was used and DBP was added to a final concentration 80 mg/l.

30 3 experiments were setup; 1)No addition of isolate , 2) Addition of isolate, 3)Addition of isolate and biomass from the biological active part of the WWTP (final concentration 3 g/l). Pure cultures of isolates a), b), and C) were tested.

The results are shown in Table 9. The result shows increased removal of DBP after ad-
35 dition of one of the isolates. In environment very similar to the activated sludge process

(3 g/l biomass) there was also an increased removal after addition of one of the isolates.

Table 9

5

	DBP removal (%)		
	Isolate A	Isolate C	Isolate D
Non bio augmented	20 ± 1	26 ± 4	24 ± 3
Bio augmented	50 ± 5	60 ± 2	63 ± 3
Bio augmented plus biomass	25 ± 5	31 ± 2	32 ± 3

Claims

1. A process for anaerobic microbial degradation of phthalic acid esters, comprising the step of adding to a bioreactor at least one bacterial strain, which as a pure isolate is capable of anaerobic degradation of phthalic acid esters.
2. The process according to claim 1, wherein the phthalic acid esters are comprised in sewage water, sludge, waste, refuse, or liquid or semi-liquid manure.
3. The process according to any of the claims 1 or 2, wherein the at least one bacterial strain is comprised in an enriched or defined culture consisting of one or more isolated strains each capable of anaerobic degradation of phthalic acid esters as a pure isolate.
4. The process according to claim 3, wherein the defined culture comprises at least two isolated strains, particularly at least three isolated strains, more particularly at least four isolated strains.
5. The process according to claim 3, wherein the enriched culture or the defined culture is supplied continuously or semi-continuously.
6. The process according to claim 5, wherein the enriched culture or the defined culture is grown aerobically prior to the addition.
7. The process according to any of the claims 3 to 5, wherein the enriched culture or the defined culture is added in an amount of at least 5 % v/v, particularly at least 10 % v/v, more particularly at least 15 % v/v, even more particularly at least 20 % v/v and the added culture comprises from 10^2 - 10^9 bacteria/ml, more particularly from 10^4 - 10^6 bacteria/ml.
8. The process according to any of the preceding claims, wherein the bacterial isolates comprise bacteria selected from the group consisting of the genera *Exiguobacterium* and *Bacillus*.
9. The process according to claim 8, wherein the *Exiguobacterium* comprises *Exiguobacterium gaetbuli* strains.

10. The process according to claim 8, wherein the *Bacillus* comprises *Bacillus fusiformis* and *Bacillus licheniformis* strains.

5 11. The process according to claim 9, wherein the *Exiguobacterium gaetbuli* strains are selected from the group consisting of DSMZ deposit number DSM 17351 and DSM deposit number DSM 17352.

12. The process according to claim 10, wherein the *Bacillus fusiformis* strain is
10 DSM deposit number DSM 17353.

13. The process according to claim 10, wherein the *Bacillus licheniformis* strain is DSM deposit number DSM 17354.

15 14. A method for isolating bacterial strains, which strains in pure form each are capable of anaerobic degradation of phthalic acid esters comprising the steps of:

a) enrichment for bacterial populations comprising bacterial strains capable of anaerobic degradation of phthalic acid esters as pure isolated strains, comprising cultivating
20 natural bacterial isolates under anaerobic conditions in the presence of phthalic acid esters under CSTR conditions;

b) isolation of single colonies on nutrient plates comprising phthalic acid esters.

25 15. The method according to claim 14, wherein the natural isolates are provided from sewage water, sludge, waste, refuse, or liquid or semi-liquid manure.

16. The method according to claim 14, wherein the CSTR is working under mesophilic anaerobic conditions for at least two hydraulic retention times.

30

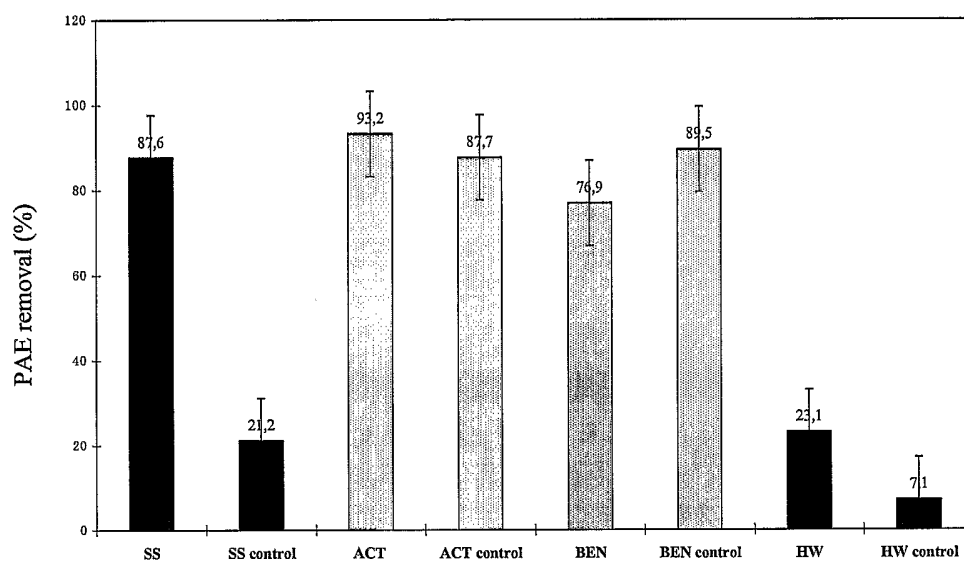
17. The method according to any of the claims 14-16, wherein the phthalic acid esters comprises Di-Butyl-Phthalate (DBP), Di-Methyl-Phthalate (DMP), Di-Ethyl-Phthalate (DEP), Di-Ethyl-Hexyl-Phthalate (DEHP), and Benzyl-Butyl-Phthalate (BBP) esters.

35

18. The method according to claim 17, wherein the phthalic acid esters comprises Di-Butyl-Phthalate (DBP) or Di-Methyl-Phthalate (DMP) esters.
19. The method according to claim 17, wherein DBP is present in the range from
5 2-1000 mg/l in the reactor, particularly from 5-500 mg/l, more particularly from 10-300 mg/l, more particularly from 20-100 mg/l.
20. The method according to any of the claims 16-19, wherein enrichment is performed for at least three hydraulic retention times, more particularly for at least four hydraulic retention times.
10
21. An isolated bacterial strain capable of anaerobic phthalic acid ester degradation and obtainable by the method according to claims 14-20, wherein said isolated bacterial strain is capable of anaerobic degradation of at least 40 % of PAE present in
15 the culture medium at concentration up to 500 mg/l, particularly at least 50 %, more particularly at least 60 %, when the said degradation is performed by the pure isolate of said bacterial strain either alone or in a defined culture of isolated strains.
22. An isolated bacterial strain capable of anaerobic degradation of phthalic acid
20 esters selected from the group consisting of DSMZ deposit numbers DSM 17351, DSM 17352, DSM 17353 and DSM 17354.
23. A composition comprising at least one isolated strain according to claim 21.
24. The composition according to claim 23, comprising at least one of the bacterial
25 strains according to claim 22.
25. A use of a bacterial strain according to claim 21 or 22, for the anaerobic degradation of phthalic acid esters.
30
26. The use according to claim 25, wherein the phthalic acid esters are comprised in sewage water, sludge, waste, refuse, or liquid or semi-liquid manure.

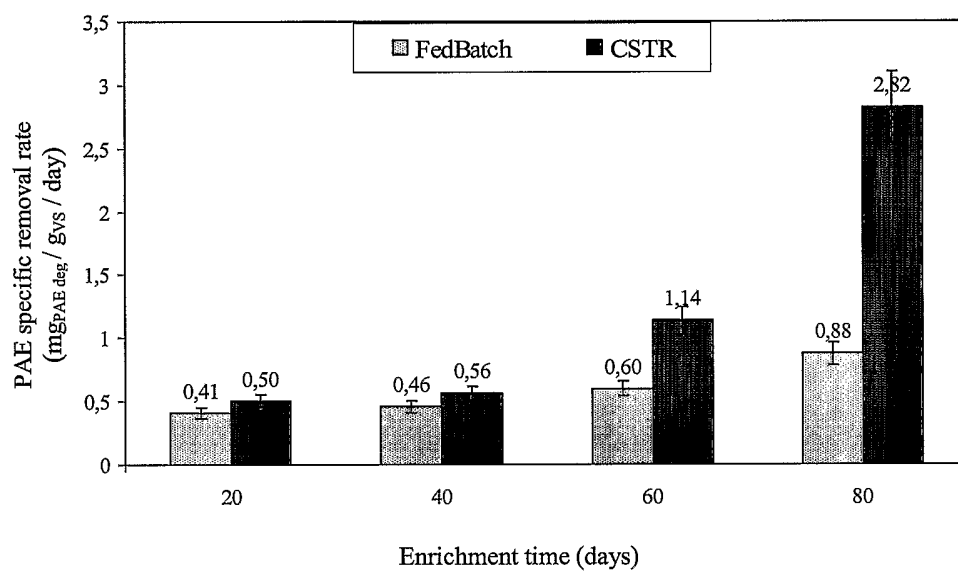
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Fig. 1



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Fig. 2



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Fig. 3a

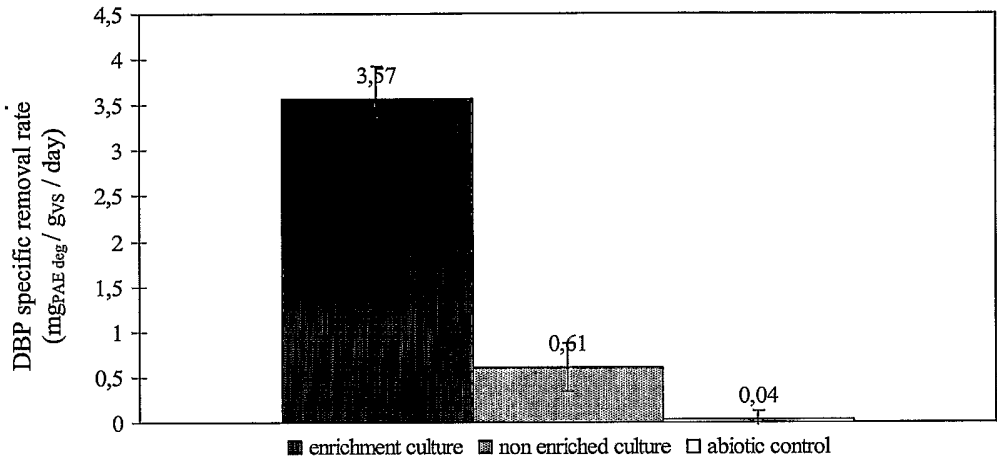
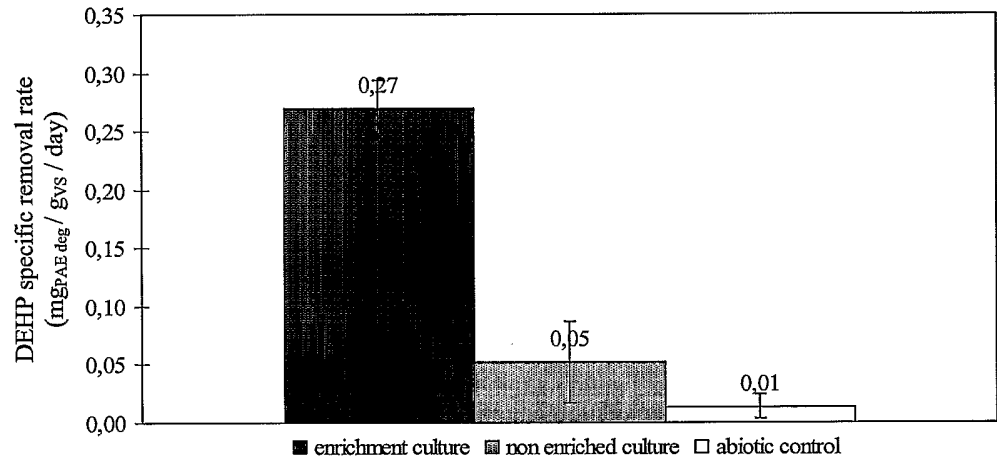


Fig. 3b



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Fig. 4a

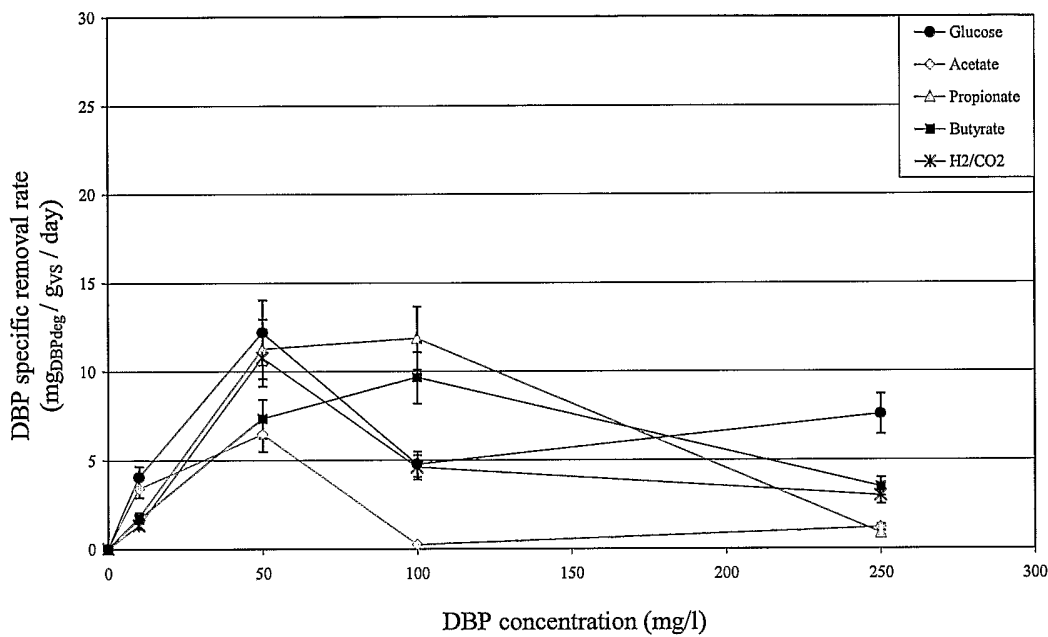
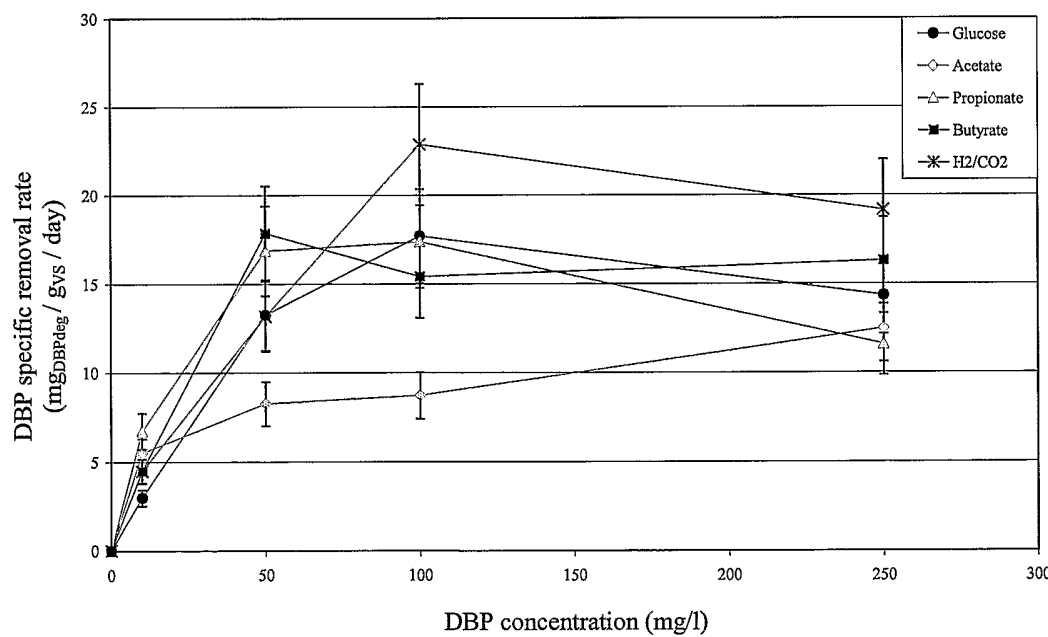
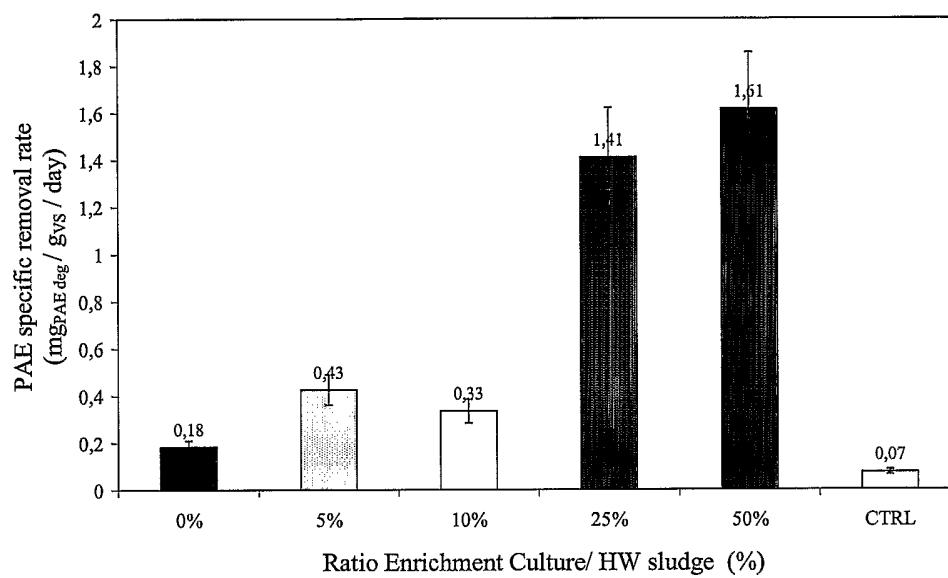


Fig. 4b



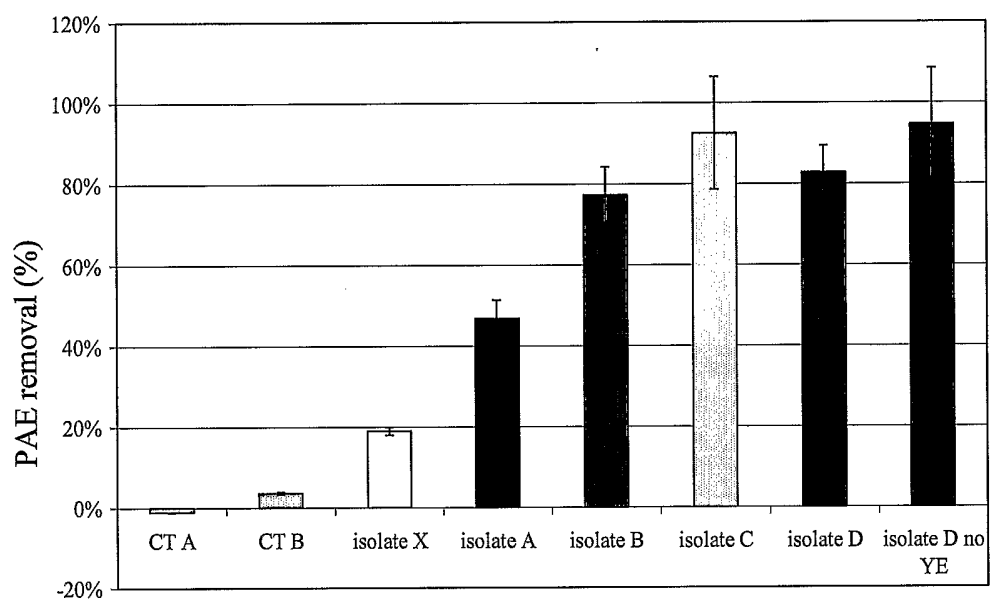
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Fig. 5



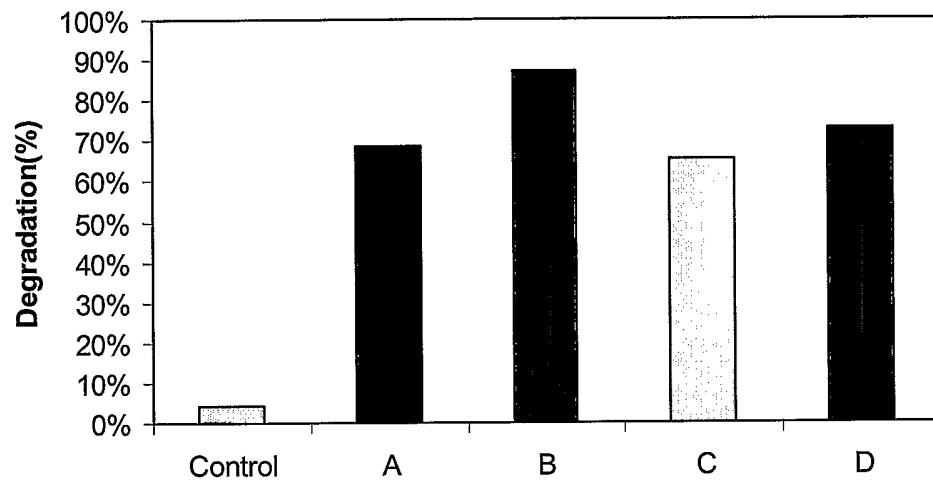
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Fig. 6



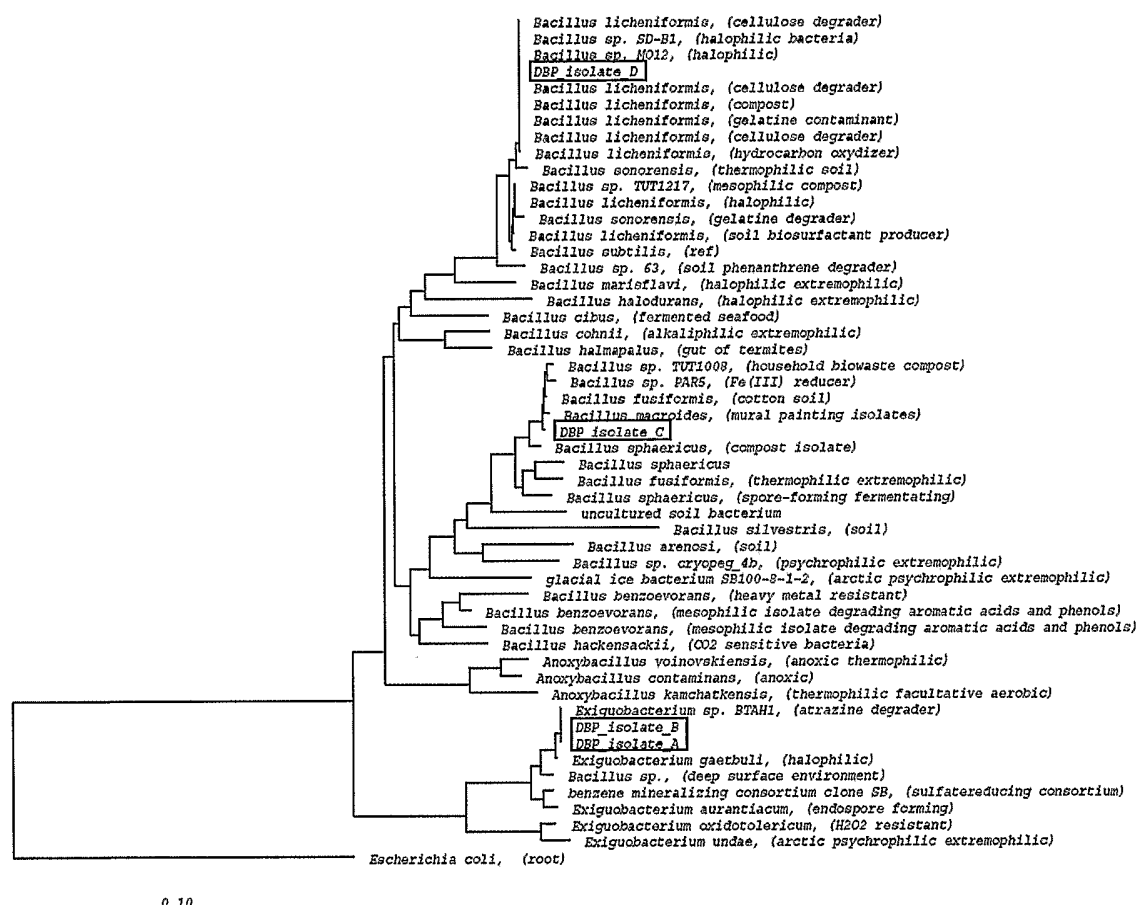
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Fig. 7



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Fig. 8



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Fig. 9

Name	Fluorescent label ^a	Target group	Formamide (%)	Non-group hits NCBI database	Probe sequence (5' to 3')	<i>E. coli</i> position	Involved in PAE degradation	Reference
SOE01	Cy3	<i>Soehngenia saccharolytica</i>	10	1 ^b	GTCATTATCTTCCCCTAG-GACAGAGC	432	YES	This study
BCT01	Cy3	<i>Uncultured Bacteroides</i> sp.	10	0	CAACCCCTTAGGGCCGCTTC	409	YES	This study
BCT02	Cy3	<i>Uncultured Bacteroides</i> sp.	10	0	GGTACCTGCAACGCTCTTATCCC G	463	NO	This study
SPYR01	Cy3	<i>Spirochaeta genus</i>	10	0	ATCCCGCGGCCATTCCCT	461	NO	This study
EUB338	Cy5	<i>Eubacteria</i>	10	0	GCWGCCACCCGTAGGTGT	338	-	Adapted from Daims et al. 1999
ARC915	Cy3	<i>Archaea</i>	20	0	GTGCTCCCCCGCCCAATTCCT	915	-	Stahl and Amman (1991)

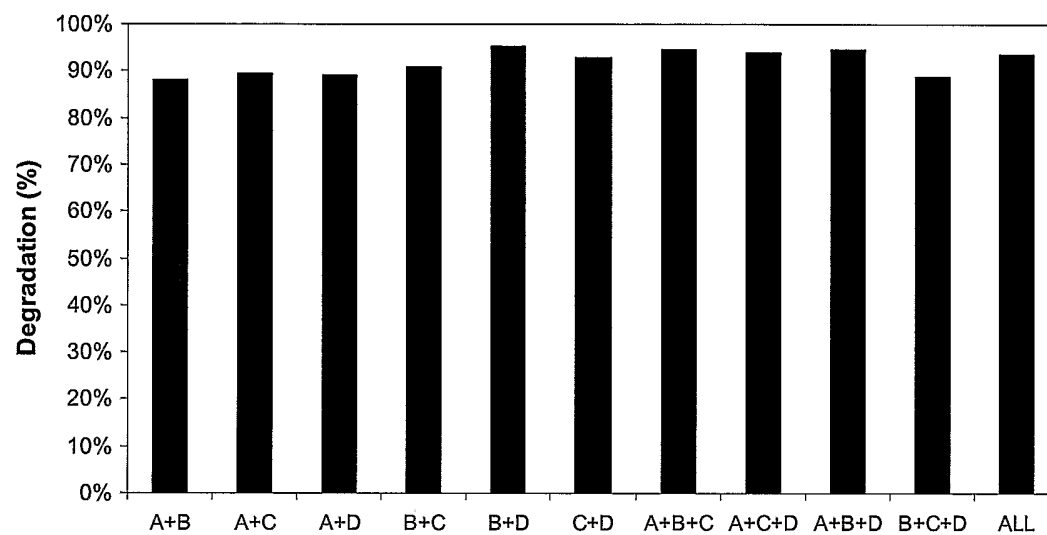
^a Cy5, indodicarbocyanine; Cy3, indodicarbocyanine^b *Catonia barnesae* (AB38361)

Name	Fluorescent label ^a	Target group	Formamide (%)	Specificity	Probe sequence (5' to 3')	<i>E. coli</i> position	Reference
ISAB-445-DBP	Cy3	<i>Exiguobacterium gaetbuli</i>	20	<i>Exiguobacterium</i> sp.	TGCCTGCGGCACCTTGTCTTCC	183	This study
ISD-775-DBP	Cy3	<i>Bacillus licheniformis</i>	20	<i>Bacillales</i> order	TTCGCTCCCCACGCTTTCGCGCT CAG	190	This study
EUB338	Cy5	<i>Eubacteria</i>	10	All bacteria	GCWGCCACCCGTAGGTGT	190	This study

^a Cy5, indodicarbocyanine; Cy3, indodicarbocyanine

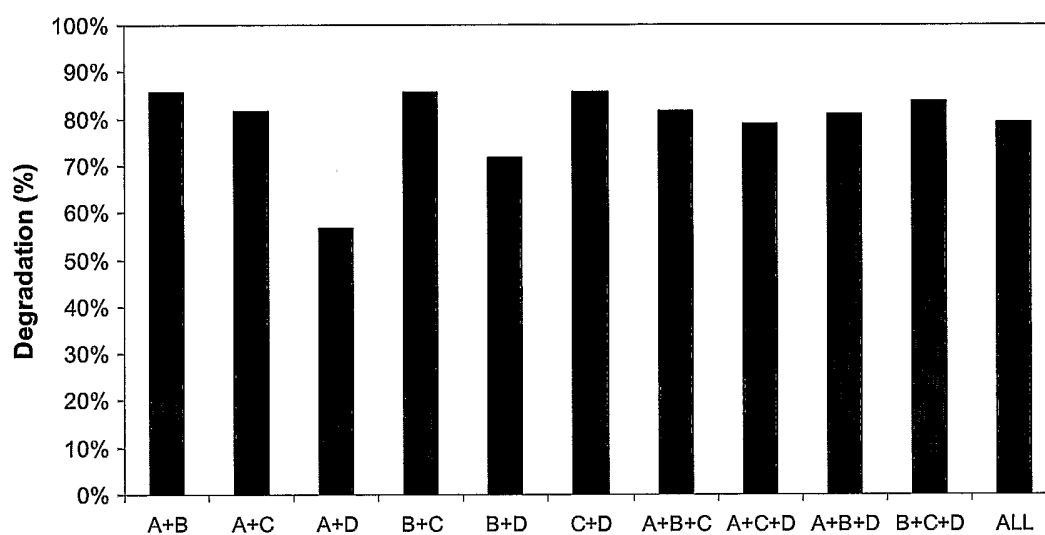
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Fig. 10



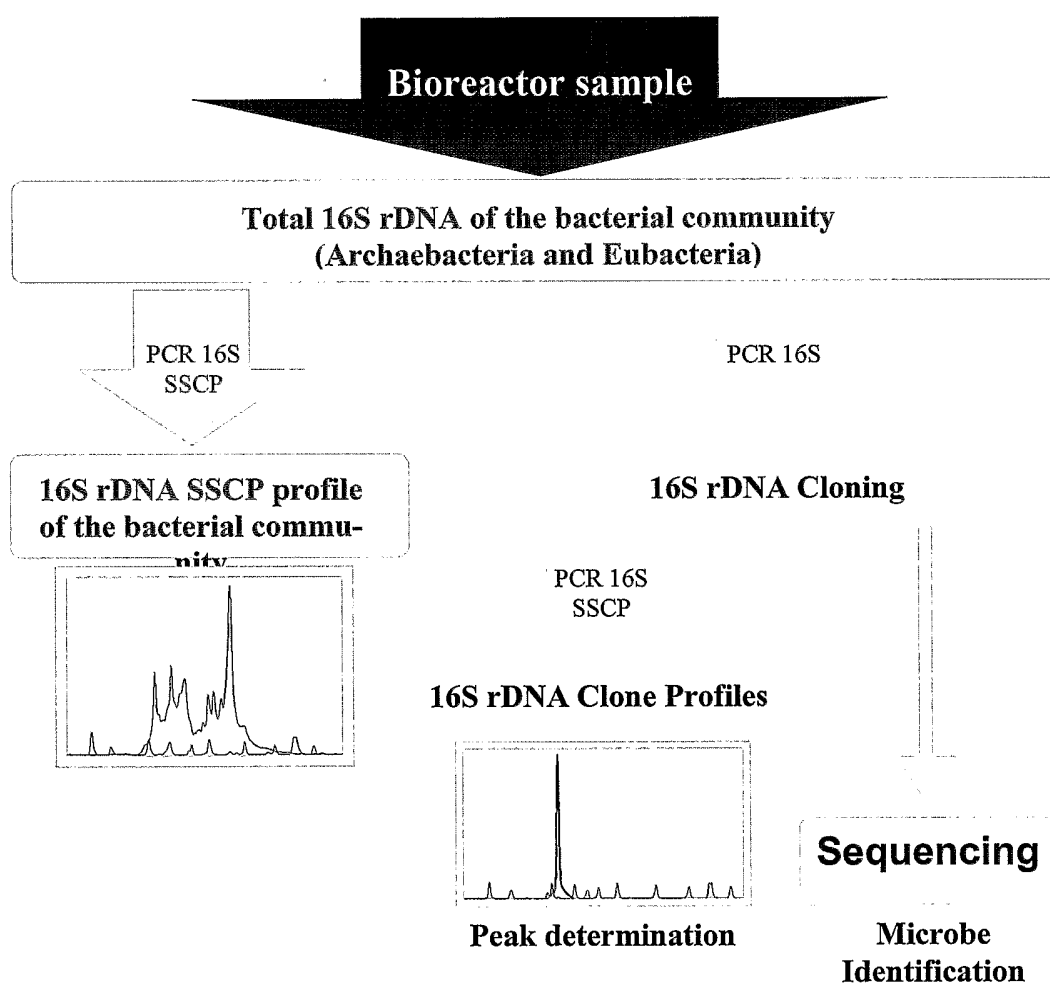
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Fig. 11



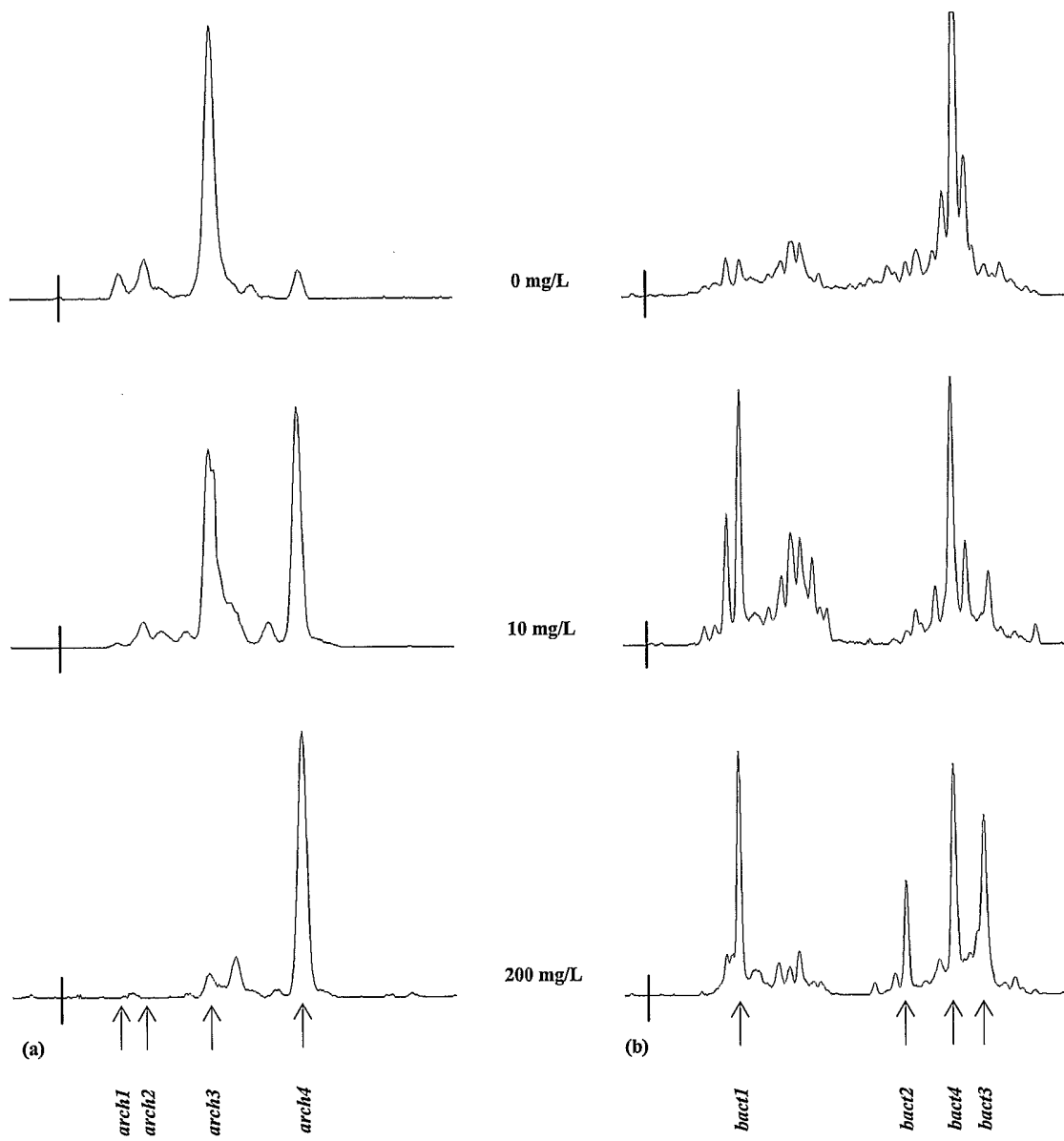
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Fig. 12



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Fig. 13



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Fig. 14

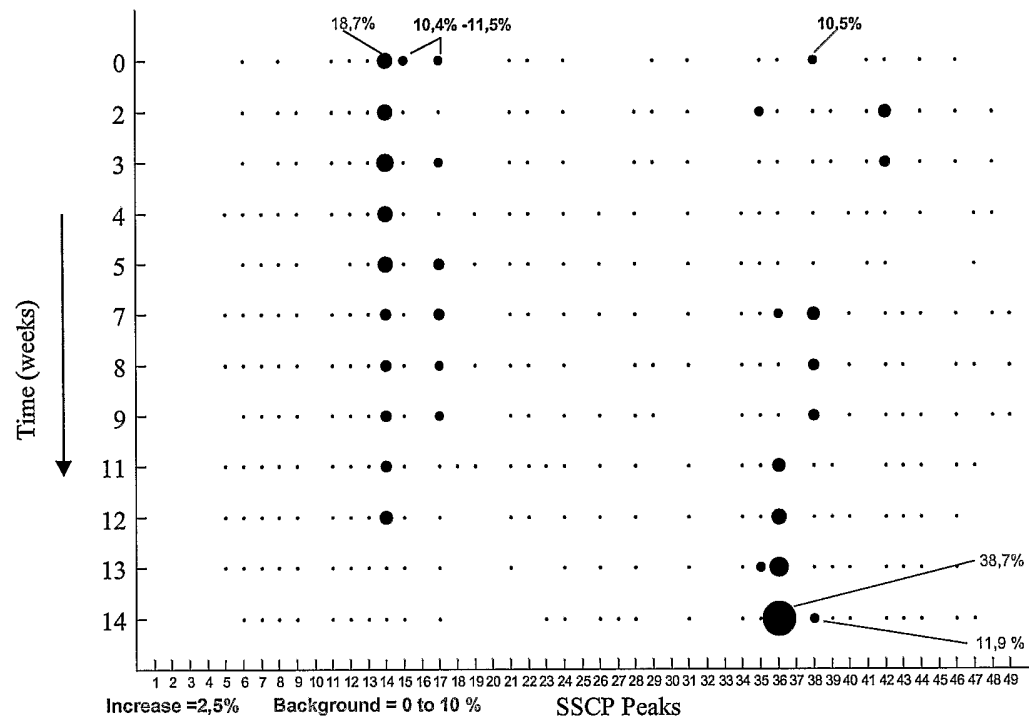
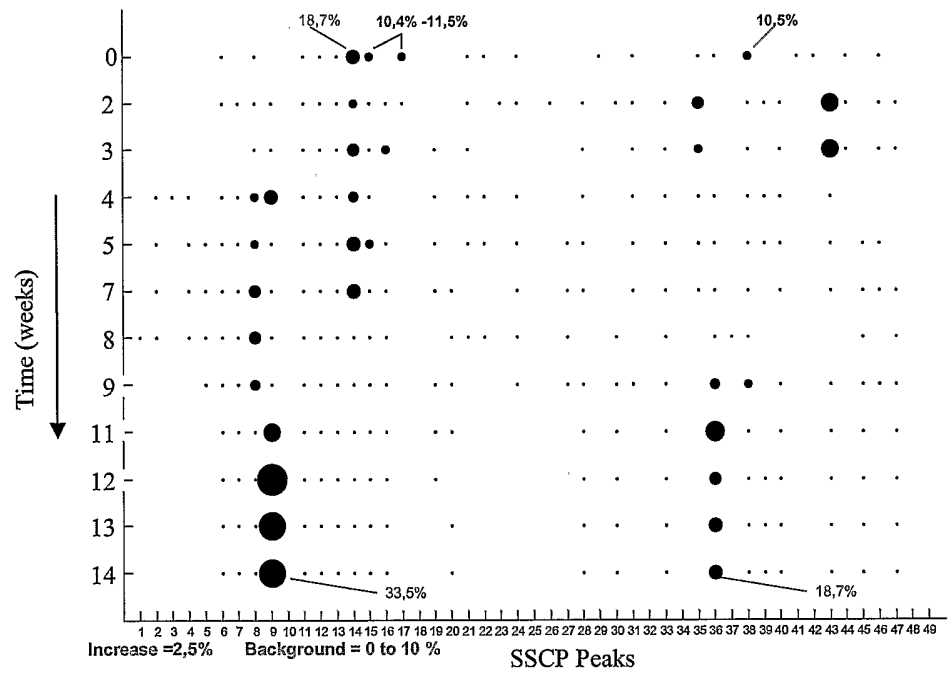


Fig. 15



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Fig. 16

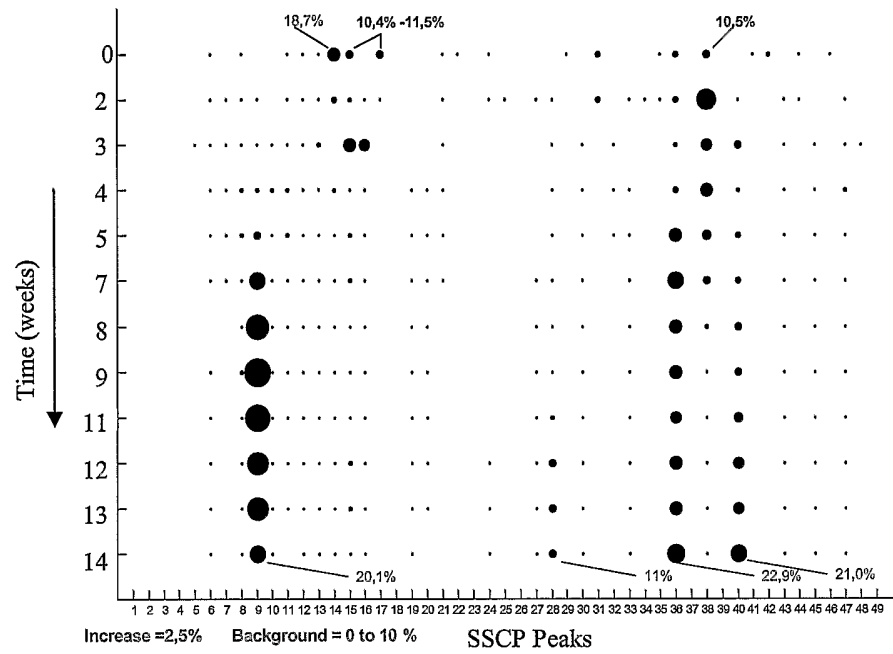
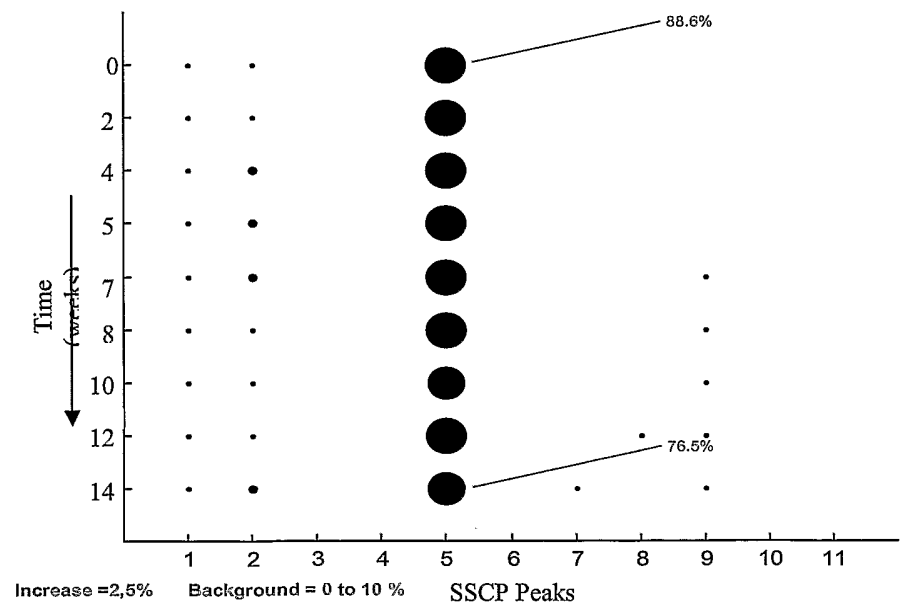


Fig 17



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Fig. 18

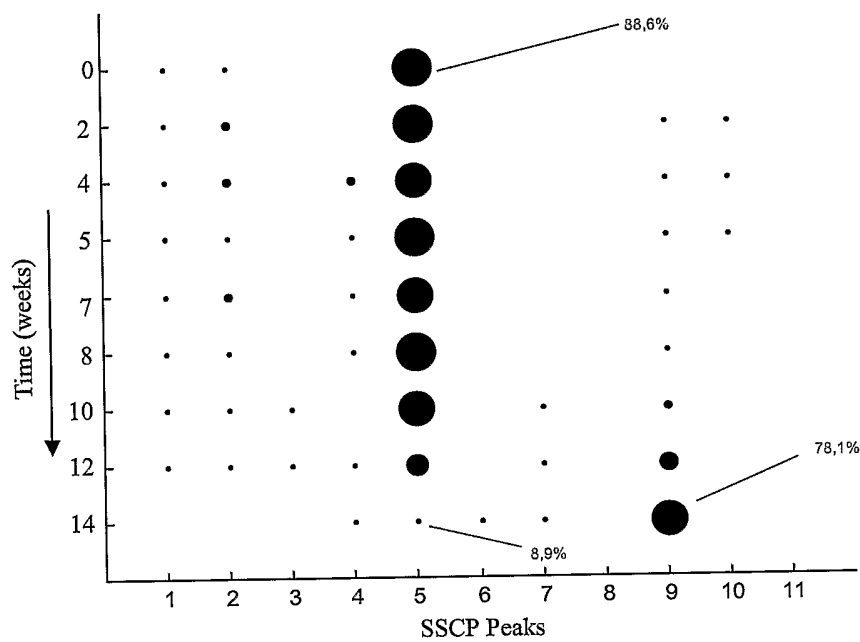
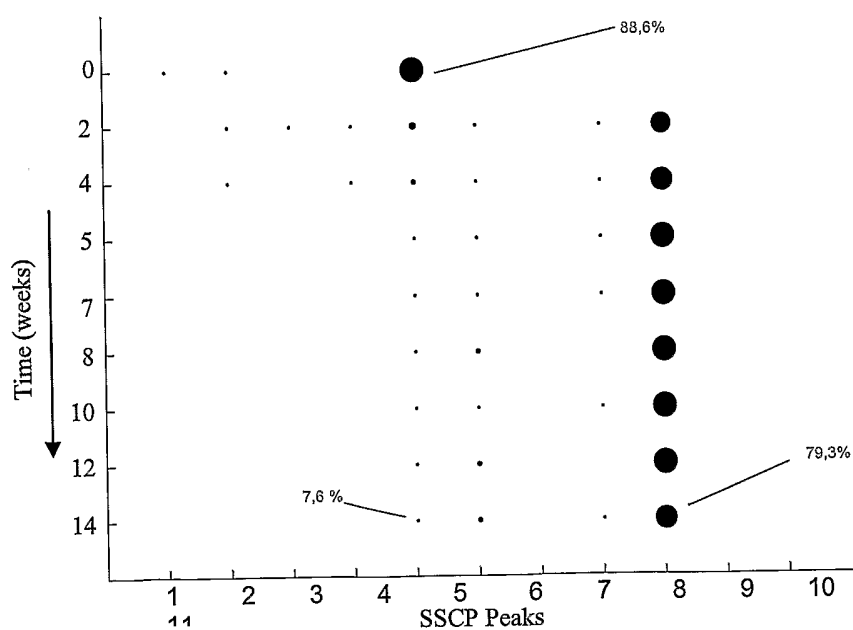


Fig. 19



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Fig. 20

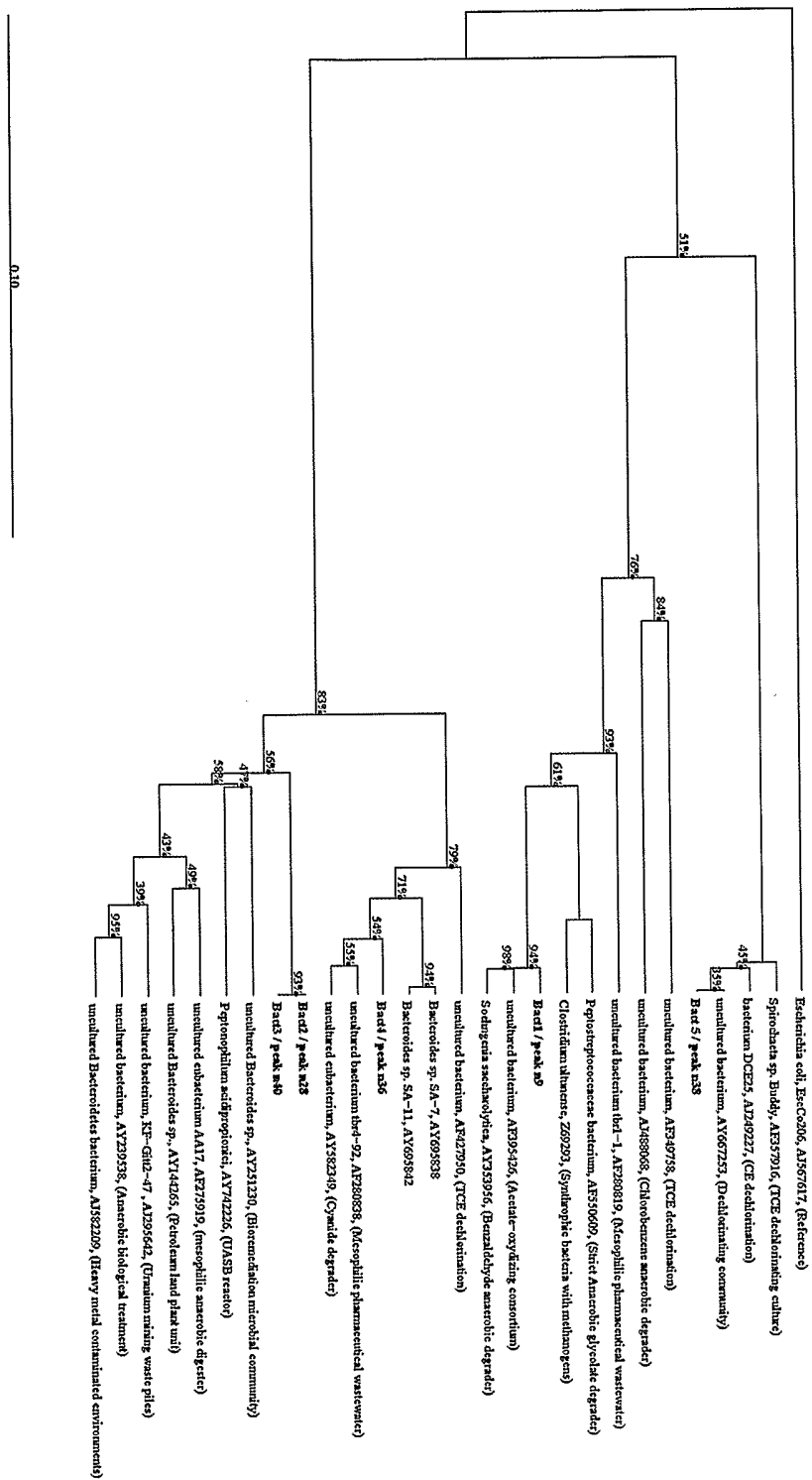


Fig. 21

