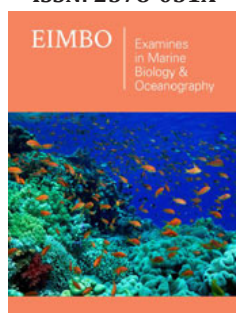


# Biofilm Surrounded Hexachlorobenzene (HCB) Crystals and Wastewater Purification

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## Abstract

Recalcitrant aromatic compounds reach the aerobic, anaerobic Wastewater Treatment Plant (WWTP) units. The outflow is discharged into receiving waters and transported into oceans. Recalcitrant aromatic compounds as Di-n-Butyl Phthalate (DBP) or HCB concerning is asked: can they serve denitrifying bacteria and archaea in WWTPs and oceans as electron donors. A special research program, financed by the German Research Foundation, tried giving an answer by adding effluent of the experimental WWTP, Civil Engineering Department, University Stuttgart, Germany, to triplicated, 50ml of a chloride-free, nitrate mineral salt medium containing 200ml flasks for enriching denitrifying bacteria and archaea. On chloride-free, nitrate containing mineral salt medium agar plates which contained *Inter alia* DBP or HCB as sole carbon source pure cultures were isolated. The with DBP or HCB as pure cultures received strains B20b1 (DBP) and B8a (HCB) served as inoculum for studying in airtight closed, the nitrate mineral salt medium containing, autoclaved, and with about  $10^7$  cells of the denitrifying, on DBP or HCB agar growing isolates inoculated flasks degradation success. About DBP has been reported [1], about the U- $^{14}$ C-labeled HCB experiments under a to 10 or 19 Vol%  $O_2$  adjusted Helium (He) atmosphere is reported here. From the in hexane dissolved added U- $^{14}$ C-labeled HCB hexane evaporated during flask evacuation and afterwards crystalized HCB covered the bottle bottom. During the 5 months of incubation (30 °C) the as *Pseudomonas aeruginosa* identified strain B 8a formed around the HCB crystals a biofilm and monthly controlled  $O_2$ ,  $CO_2$ ,  $N_2O$  and  $N_2$  changes in the flask atmosphere, the from the originally added 156mg HCB as  $^{14}CO_2$  released  $0.084 \pm 0.3$ mg into the flask headspace, and the  $13.26 \pm 1.03$ mg accumulated U- $^{14}$ C-HCB in cells and biofilm of the 10%  $O_2$  flask variant signaled HCB interactions which are discussed.

**Keywords:** Tertiary wastewater treatment; *Pseudomonas aeruginosa*; Denitrifying wastewater effluent isolate; Hexachlorobenzene degradation; Biofilm formation

## Introduction

Wastewater aeration is the first step in achieving optimal conversion of the WWTP reaching organic,  $NH_2$ -substituted compounds into  $CO_2$  and  $NO_3^-$  by  $O_2$  respiration and nitrification. The pre-purified wastewater is as next step channeled through a non-aerated zone to reduce the formed  $NO_3^-$  load in the first purification step largely to  $N_2$  with the not  $O_2$  respired, leftover organic compounds as carbon source [2]. The arising question is now: are  $O_2$  respiration withstanding recalcitrant organic compounds suitable carbon sources for the denitrifying wastewater community? For giving an answer the Civil Engineering Department of the University Stuttgart, Germany, initiated a special, by the German Research Foundation financed research program in which the soil microbiology unit of the Soil Science Department, University Stuttgart-Hohenheim, has tested whether compounds as DBP or HCB are suitable electron donors for nitrate respiration. If not, such compounds will accumulate in the forming sewage sludge or are discharged into the receiving water by finally reaching the oceans. After volume reduction in digesters the sewage sludge is deposited in dumps, spread in often soil nutrient buffer capacity surpassing amounts together with animal slurries from industrial livestock farming on a limited land area, or burnt [3].

Already the Romans realized around 100 years before our calculation of time that wastewater can create problems and connected households to a sewer network. Forced by a growing population and industrialization the German capital Berlin hired 14.364 Hectares

(Ha) land in 1913 and installed a sewage farm on which Berlin's wastewater was spread (1988 still to 34%). In 1926 Berlin's industry contributed to the discharged wastewater 7.3% and from the continuously with nutrients enriched sewage farm health menacing amounts of side drained  $\text{NO}_3^-$  reached the glacial valley wells, groundwater, and the surface water (riverbank filtrate), Berlin's drinking water resources. Such contaminations are meanwhile worldwide observed and the Civil Engineering Department of the University Stuttgart, Germany, initiated in the 1970ies a wastewater onsite treatment improving research program with the aim to get new insights in wastewater purification [4]. Concomitantly the U.S. Environmental Protection Agency (USEPA) and other administrations issued detailed guidance for onsite wastewater treatment systems (OWTSs; Design Manual: Onsite Wastewater Treatment and Disposal Systems, USEPA, 1980) and we started with lyophilized effluent of the experimental wastewater treatment plant (Civil Engineering department) by adding the lyophilized effluent powder in a concentration of 0.2% to an artificial, nitrate containing mineral salt medium agar for enriching on recalcitrant organic compounds pre-adapted denitrifying bacteria strains. Textbooks at the time of experimentation stated that aromatic nuclei are preferred (exclusively) aerobically cleaved, but instead we were successful in bringing the on di-n-butyl phthalate agar growing strain B20b1 and the on HCB agar growing strain B8a in pure cultures [1,5].

The experiments with strain B20b1, deposited at the Sweden type culture collection are described [1] that with ( $\text{U-}^{14}\text{C}$ )-labelled HCB is discussed here. Promising to start with ( $\text{U-}^{14}\text{C}$ )-labelled HCB experiments was a reported degradation experiment with hexachlorocyclohexane [6]. A job offers from the International Rice Research Institute, Los Banos, Philippines, at the end of my doctorate (early 1980) to study the phenomenon of iron toxicity with rice prevented to publish the findings with HCB isolate strain B8a earlier, also the circumstance that in spite of made arrangements after my returning to the Stuttgart department the strain was lost. In the 40 years to today transformation experiments with relatively recalcitrant aromatic compounds, even with HCB and under denitrifying conditions, have been repeatedly reported and the redox potential cascade in biofilms is much better understood [7-20]. Meanwhile is known that a self-produced, by an extracellular matrix surrounded biofilm makes in hostile environments microorganism communities more resistant and all that encourages to re-discuss the observations made with the wastewater concentrate isolate strain B 8a and HCB and to speculate about oceans and sewage sludge problem solutions.

## Material and Method

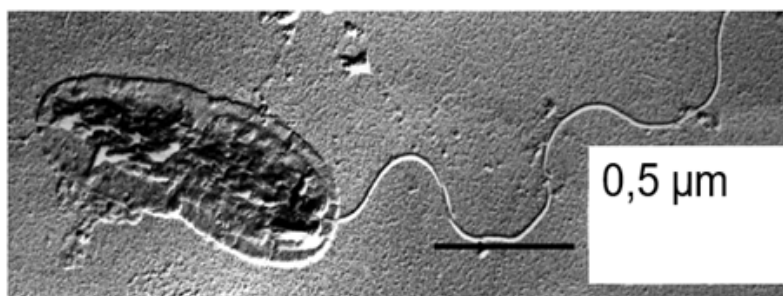
### Denitrifying bacteria enrichment

About 100 liters effluent of the experimental wastewater plant Civil Engineering Department, University Stuttgart, Germany, were condensed in a double-walled steel container, through to  $-15\text{ }^\circ\text{C}$  cooled methanol flow. The condensed effluent was lyophilized, and the obtained concentrate was characterized by determining the ignition loss (ash content), the trace elements with a flame

atomic absorption spectrometry (FAAS; Perkin-Elmer Model 303; [21]) and the fatty acids with a Flame Ionization Detector (FID) in a HP 6890 gas chromatograph [22] (carrier gas  $\text{N}_2$  30ml  $\text{sec}^{-1}$ ; HP-88 INNOWAX detector, carrier gas  $\text{H}_2$ , 40ml  $\text{min}^{-1}$ , make-up gas air 450ml  $\text{min}^{-1}$ ; Agilent 5183-4647-columns: Model Nr. 112-88A7, 100m x 0.25mm ID, 0.2 $\mu\text{m}$ , Model Nr. 19091N-133, 30m x 0.25mm ID, 0.25 $\mu\text{m}$ ; column injector, detector temperature 250  $^\circ\text{C}$ ). The such characterized effluent powder was in a 0.2% (w/v) concentration added to a chloride free, nitrate containing mineral salt medium and to agar plates for preselecting and enriching denitrifying, on not easily degradable compounds pre-adapted bacteria ( $\text{Na}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{KNO}_3$ ,  $\text{NH}_4\text{OH}$ ,  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ ,  $\text{CaSO}_4$ ,  $\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$ ,  $\text{Co}(\text{CH}_3\text{CO}_2)_2 \times 4\text{H}_2\text{O}$ ,  $\text{Mn}(\text{CH}_3\text{COO})_2$ ,  $\text{C}_6\text{H}_8\text{O}_7 \cdot n\text{Fe} \cdot n\text{H}_3\text{N g L}^{-1}$ , 11.2, 0.8, 6.0, 1.0, 0.2, 0.002, 0.001, 0.001, 0.001, 0.005g  $\text{L}^{-1}$  dist. water, 15g agar-agar  $\text{L}^{-1}$ , pH 7.4, respectively, [23,24]). During incubation in an anaerobic jar under a 9:1  $\text{N}_2\text{-CO}_2$  atmosphere up growing colonies were fractionated streaked out on fresh agar plates which instead of effluent powder contained the frequently used non-ionic emulsifier polyethylene sorbitane monooleate (Tween 80), phthalate based aromatic structures, trichlorotoluene, 1,2, 1,3, 1,4 dichlorobenzene, 1,2,4 trichlorobenzene,  $\gamma$ -hexachlorocyclohexane, or HCB as sole carbon source. Up growing colonies were sub-cultivated until morphologically uniform shaped cell structures became visible under the microscope.

### Identification of the on HCB containing agar plates isolated strain B 8a

Under the microscope as the morphologically uniform shaped strain B 8a cells were for describing morphology and flagellation type sputtered with Pt/Ir 45 $^\circ$  for making the cells visible under the transmission electron microscope Siemens 80 kV: (Figure 1). For getting some genomic information DNA was extracted with Na-dodecyl sulfate and with lysozyme the DNA-strands separated. The DNA guanine, cytosine Mol% was estimated in a temperature-controlled cuvette by the melting point method ( $T_m\text{-}53.9 \times 2.44$ ; DNA absorbs UV light of 260nm, [25]). As further identification criteria the metabolic potential served: growth at 4 and 41  $^\circ\text{C}$ , oxidation of gluconate, aromatic ring cleavage, gelatinase, lecithinase, amylase activity, levane formation, the capability to gain energy with trehalose, inositol, geraniol, or acetamide and the strain B 8a properties of secondary taxonomic value as gaining energy with sugars (D-fructose, D-glucose, L-arabinose, 2-ketoglutarate, starch), alcohols (ethanol, adonitol, sorbitol, glycerol), organic acids (propionate, butyrate, myristic acid), aromatic, cyclic (chinate, protocatechuate, benzoate, p-, m-OH benzoate, benzole, phenylacetate, anthranilate, benzylamine, Hippurate), and N containing compounds (glycine,  $\beta$ -alanine, arginine, amino valerate, keratin, betaine of strain B 8a were tested and the antibiotic sensibility with the antibiotics: gentamycin, kanamycin, neomycin, novobiocin, paromomycin, rifamide, rifampicin, rifamycin, sulphatriad, cephalixin, cephalotin, erythromycin, nitrofurantoin, ampicillin, bacitracin, methicillin, oxacillin, penicillin g, polymyxin, b colistin sulfate, fusidin acid, doxycycline, tetracycline, carbenicillin, chloramphenicol, and clindamycin caused by inhibition zones [26-28].



**Figure 1:** TEM picture of the 80.000 times magnified denitrifying strain B 8a with polar flagellation and identified according to Stanier et al. [44] as *Pseudomonas aeruginosa*.

### (U-<sup>14</sup>C)-labelled HCB experiment

For giving an answer on the question: can denitrifying bacteria grow with the organic Compound HCB as electron donor 50ml of a chloride-free mineral base medium were enriched with 700µg ml<sup>-1</sup> NO<sub>3</sub><sup>-</sup>-N and filled in triplicated 200ml Erlenmeyer flasks. After autoclaving (10min, 121 °C) the flasks were air-tight closed with rubber stopper and with sterile syringes in 2.5ml hexane dissolved 7.5mg cold and 0.3mg (U-<sup>14</sup>C)-labelled HCB and about 10<sup>7</sup>, denitrifying, 3 times in sterile water pre-washed strain B 8a cells (Figure 1) were added. Afterwards the flask atmosphere was 5 times replaced by filtered helium (purity 99.9, Messer Griesheim) and adjusted to an O<sub>2</sub> (v/v) concentration of 10 or 19%. All not-inoculated and HCB-free control flasks and those which were inoculated and contained (U-<sup>14</sup>C)-labelled HCB were incubated in an anaerobic jar under a 9:1 N<sub>2</sub>-CO<sub>2</sub> atmosphere as recommended for food preserving for 5 months. During flask evacuation the HCB solvent hexane evaporated, and HCB crystals covered the flask bottom (Figure 2). In the flask atmosphere monthly with the Gas Chromatograph (GC), model F22, equipped with a Thermal Conductivity (TCD) to an Electron Capture (ECD) connected detector (Perkin-Elmer, Überlingen, Germany) the CO<sub>2</sub>, N<sub>2</sub>O, O<sub>2</sub> and N<sub>2</sub> changes were controlled. The gas samples passed after injection a Poropak-Q packed, stainless-steel column and for separating O<sub>2</sub>, N<sub>2</sub> a 5A molecular sieve packed column (helium flow 20ml min<sup>-1</sup>, column temperature, 54 °C, injector, 54 °C, detector 150 °C, and filament 300 °C). The peak areas were integrated with the Perkin-Elmer GC system SIP-1.

With help of a vacuum pump the flask atmosphere was sucked after the 5 months of incubation through 3 in line connected washing filters and the CO<sub>2</sub> collected in 70ml of a 0.1N NaOH. In 1,2,4 trimethyl benzene scintillator (Packard Insta Solve, nr 6003183), into which 0.1N NaOH proportions were transferred the <sup>14</sup>CO<sub>2</sub> was quantified with a Packard Tri Carb Liquid scintillation spectrometer (Model 3380). Since the liquid flask phase could not be alkalized out of NO<sub>3</sub><sup>-</sup>-N interference reasons, the assumed HCB conversion products in the mineral salt medium were extracted 3 times with 50ml hexane. Afterwards the hexane (150ml) was passed through a 2µ sterile filter for separating liquid phase and the grown bacteria, biofilm biomass. The <sup>14</sup>C share on the with 50ml hexane rewash filters was converted into CO<sub>2</sub> with an incinerator Packard Tri Carb sample oxidizer (Model 306) that was transferred into carborb

scintillator (Packard) and after injection into a Packard Tri Carb Liquid scintillation spectrometer (Model 3380) quantified [29,30]. The <sup>14</sup>C share in liquid flask phase was quantified by adding 10ml 0.1N NaOH to 20ml of the 150ml hexane extract. Aliquots of the into the eventually HCB conversion products containing NaOH phase were injected into the Packard Tri Carb Liquid scintillation spectrometer (Model 3380) and quantified. From the inoculated variants the <sup>14</sup>C radioactivity, measured in the controls, was subtracted and <sup>14</sup>C in biofilm, cells, the liquid phase and the <sup>14</sup>CO<sub>2</sub> in 150ml flask atmosphere expressed in mg. Eventually liberated chloride from the HCB crystals was analyzed titrimetrically [31].

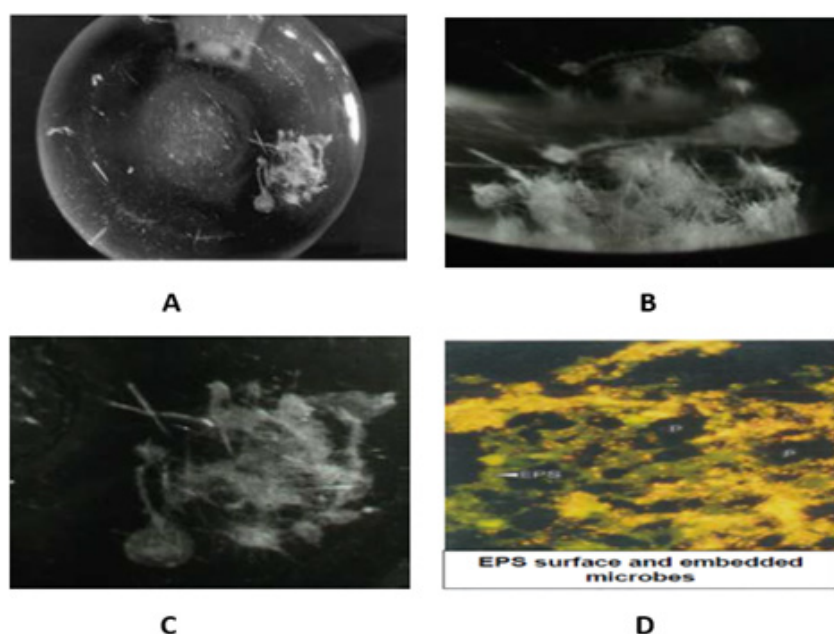
All used chemicals were of analytical grade and purchased from Merck-Schuchardt, Germany. The from Messer Griesheim, Germany, purchased He had a purity of 99.998% and the by the Hoechst company provided (U-<sup>14</sup>C)-labelled HCB a specific activity of 9.0Ci g<sup>-1</sup> and a radiochemical purity >> 99%. The water of the 50ml culture medium could have retained after evacuation hexane in an amount of maximum 0.5 mg [25 °C] [32].

### Result

Strain B 8a formed a green-blue phenazine pigment as described for *Pseudomonas aeruginosa* strains deposited in type culture collections was, monotrich, polar flagellated (Figure 1) and had a Guanine, Cytosine (GC) Mol% of 66.8 [33-36]. These of primary taxonomic value differentiating characteristics supplement the tested physiological capabilities and antibiotic sensibilities of strain B 8a that grew at 41 °C not at 4 °C, could oxidize gluconate, cleave ortho-protochatechuate and other aromatic ring structures, and showed a positive lecithinase, esterase reaction, but a negative amylase and variable gelatinase reaction. Tolerated was cetrimide and energy could be gained with geraniol and acetamide, D-glucose, 2-ketogluconate, ethanol, adonitol, glycerine, propionate, butyrate, myristic acid, polyethylenesorbitanemonooleate (Tween 80), chinate, protochatechuate, benzoate, p-OH benzoate, anthranilate, glycine, β-alanine, arginine, aminovalerate, and betaine. On the with Oxoid disks in a concentration of 10, 300, 30, 30, 300, 10, 300, and 100µg offered antibiotics gentamycin, doxycycline, rifampicin neomycin, sulphatriad, colistinsulfate, polymyxin B, and carbenicillin reacted strain B 8a with a bacteria-free areola of 14, 6, 6, 4, 8, 4, 6, 9mm, respectively. Based on all these tests strain B 8a (Figure 1) belongs very likely to the *P aeruginosa* family.

Whether strain B 8a (Figure 1) would be able to use the 6-fold chlorinated HCB as carbon source as tested in a ( $^{14}\text{U}$ )-labelled HCB experiment showed at first that strain B 8a started to form a biofilm around the ( $^{14}\text{U}$ )-labelled HCB crystals around 2 months after incubation (Figure 2). The monthly in the flask atmosphere measured  $\text{O}_2$  decreases and  $\text{CO}_2$ ,  $\text{N}_2\text{O}$  and  $\text{N}_2$  releases into the  $\text{O}_2$ /helium atmosphere (Figure 3), the  $^{14}\text{CO}_2$  released in significantly lower concentration, and the  $^{14}\text{C}$  in the liquid mineral salt,  $\text{NO}_3^-$ -containing medium phase and in the *P aeruginosa* cells and biofilm (Table 1) signalized interaction between HCB and strain B 8a. The GC measured  $\text{O}_2$  concentration in the flask atmosphere of the 10%  $\text{O}_2$  variant decreased from about 10 to 4% and in the 19%  $\text{O}_2$  variant from 19 to 14%. With  $\text{O}_2$  decrease in the flask atmosphere of the 10%  $\text{O}_2$  variant  $\text{N}_2\text{O}$  increased to almost  $80\text{mg L}^{-1}$ , in the 19%  $\text{O}_2$  variant to around  $10\text{mg N}_2\text{O L}^{-1}$  (Figure 2). Reverse was the observation with  $\text{CO}_2$  that accumulated in the flask atmosphere of

the 19%  $\text{O}_2$  variant to about  $50\text{mg CO}_2 \text{L}^{-1}$ , in the 10%  $\text{O}_2$  variant to only  $20\text{mg CO}_2 \text{L}^{-1}$ , whereas the  $^{14}\text{CO}_2$  release into the flask atmosphere of 10% 19%  $\text{O}_2$  variant was with 0.072 and 0.060mg almost similar (Table 1). About the  $\text{CO}_2$  release differences could be speculated that they may rely on not evaporated metabolized hexane but more likely on metabolized dead strain B 8a cells, used by the survivor cells as carbon source and that better at a higher  $\text{O}_2$  availability [37,38]. The addition of wastewater concentrates and a higher  $\text{O}_2$  availability was biofilm formation and HCB accumulation concerned rather preventing than supporting (Table 1). The high Zn concentration of  $1740.4\mu\text{g g}^{-1}$ , combined with 113.1, 102.6, 9.5, 2.3 Cu, Ni, Pb, Cd  $\mu\text{g g}^{-1}$  (0.3, 0.03, 0.03, 0.002, 0.001% of the ash content) in the effluent concentrate and the nearly 77% lower nitrate conversion in the 19%  $\text{O}_2$  partial pressure variant than in the 10%  $\text{O}_2$  (v/v) variant could be an explanation (Table 1; [39,40]).



**Figure 2:** Strain B 8a biofilm after 5 months of incubation in a nitrate and HCB containing mineral salt medium (A,B,C) and D [16].

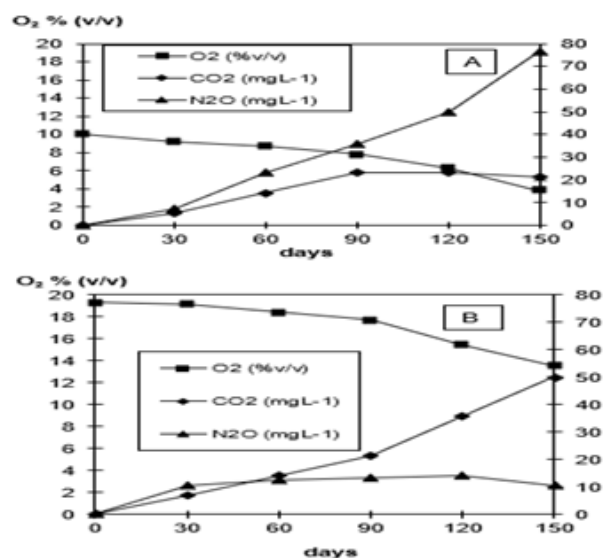
**Table 1:**  $\text{U-}^{14}\text{C}$ -hexachlorobenzene transformation in presence of *Pseudomonas aeruginosa* B 8a in a chloride-free mineral salt medium (pH 7.4) after a 5-month incubation ( $30^\circ\text{C}$ ) at 2 different  $\text{O}_2$  partial pressures<sup>®</sup> and with and without  $40\text{g L}^{-1}$  concentrate of the wastewater treatment plant effluent, model plant Civil Engineer Department, University Stuttgart, Germany (U).

A. Values are the mean of 3 parallels minus controls

B. Original HCB content was  $7.5\text{mg cold}$ ,  $0.3\text{mg (U-}^{14}\text{C)}$ -labelled HCB per flask.

C. The used wastewater concentrate added to separate flasks with cold (U- $^{14}\text{C}$ )-HCB contained  $388.2\text{mg chloride}$ .

$^{14}\text{C}$ Balancing ( $\text{mg } 200\text{ml}^{-1} \text{Flask}^{-1}$ )						
Initial $\text{O}_2$ Vol %	Chloride waste release water without Biofilm	$^{14}\text{CO}_2$ in the flask pressure with Biofilm	$^{14}\text{C}$ in hexane phase	$^{14}\text{C}$ in crystal hexane phase	Flask $^{14}\text{C}$ +HCB atmosphere	Concentrate
10.9	$1.5\pm 0.3$	0.0036	$0.084\pm 0.3$	$1.33\pm 1.03$	$7.8\pm 1.7$	no addition
19.3	$0.64\pm 0.26$	0.003	$0.048\pm 0.058$	$0.0146\pm 0.23$	$7.8\pm 1.6$	no addition
10.9	$50.8\pm 16.6$	0.0018	$0.12\pm 0.08$	$1.12\pm 0.25$	$7.8\pm 1.9$	$0.34\pm 0.12$
19.3	$47.5\pm 9.5$	0	$0.002\pm 0.017$	$1.3\pm 0.23$	$7.8\pm 5.6$	$0.26\pm 0.11$



**Figure 3:** O<sub>2</sub> decrease and CO<sub>2</sub>, N<sub>2</sub>O and N<sub>2</sub> release at 2 different O<sub>2</sub> partial pressures into the helium atmosphere during a 5-month incubation of strain B 8a, identified as *Pseudomonas aeruginosa*, in presence of HCB as only carbon source.

## Discussion

From the estimated to  $1.2 \times 10^{30}$  prokaryotic cells on Earth roughly  $4 \times 10^{29}$  survive in deep oceanic sub-surfaces,  $5 \times 10^{28}$  in upper oceanic sediments,  $3 \times 10^{29}$  in deep continental sub-surfaces, and  $3 \times 10^{29}$  in ocean surface microlayers, the phyllo- and atmosphere, the groundwater, in solid soil particles surrounding water films, and in, on human, animal bodies [17]. For guaranteeing a better surviving prokaryotes have invented the capability to conserve energy by O<sub>2</sub> respiration, anaerobic respiration, and fermentation and to form phenazines and biofilms [41,42]. Most of these capabilities *P. aeruginosa* strains have for their disposal. They can conserve energy by O<sub>2</sub> and NO<sub>3</sub><sup>-</sup> respiration and have a remarkable adaptability on pollutants and antibiotics through phenazine and biofilm self-production (Table 1; Figure 2) [27,43-46]. Biofilms consist out of nucleic acids, polysaccharides, lipids, proteins, outer membrane proteins and to approximately 30% add of the proteins go back on biofilm attached microbes [47,48]. A polymeric substance (EPS) layer surrounds a biofilm that consists to about 97% out of water, extracellular DNA (eDNA), gel-forming polysaccharides, proteins, insoluble amyloids, and cellulose. EPS facilitates the liquid transport into the biofilm and

**Table 2:** Characterization of the lyophilized wastewater concentrate, experimental wastewater treatment plant effluent, Civil Engineer Department, University Stuttgart, Germany

A. Caprylate (6.2%), laurate (2.8%), myristic acid (5.9%), palmitic acid (20.1%), palmitoleic acid (20.6%), stearic acid (17.0%), oleic acid (22.4%), linoleic acid (4.3%), arachidic acid (0.3%).

B. Zn, Cu, Ni, Pb, Cd 0.3, 0.03, 0.03, 0.002, 0.001%, respectively, the relatively high Zn% can cause toxic effects.

Lyophilised Wastewater Concentrate	Ignition Loss Organic Content	Inorganic Content	Raw Fat Content	Fatty Acid Content	% Trace Element of Inorganic Content mg L <sup>-1</sup> μg L <sup>-1</sup>
576±20	167±2	408.3	75.8	0.97 <sup>A</sup>	0.363% <sup>B</sup>
29.10%	70.90%	13.20%	0.20%		

the escaping from the biofilm [37,49]. Inside of biofilms living pro- and eukaryotic Geno- and phenotypes sustain a wide range of physiological states (redox potentials) and distinct metabolic pathways [50-53]. Possess biofilm inhabiting or on the EPS layer attached planktonic cells as strain B 8a (Figure 1) fimbriae, pili, or a flagella the escaping from the biofilm into a growth and surviving better supporting outside environment is easier, but for escaping a DNA-specific endonuclease End A that degrades eDNA is needed [54-56].

Into mature, in average 210μm thick biofilm oxygen penetrates to a depth of about 50μm and the large anoxic biofilm region beyond the O<sub>2</sub> zone narrows the growth rate of there residing bacteria, archaea, fungi, protozoa compared with logarithmic growing planktonic cells drastically to 0.02h<sup>-1</sup> [57,58]. Thus, biofilm surround or attached planktonic cells will first enter into logarithmic growth when they have left the biofilm interior or EPS layer attached position. The biofilm interior inhabiting microbial cell types are clustered in micro-colonies and surrounded by a mechanically stabilized, specific living environment (Figure 2) that after having perceived an eDNA constituted signal starts developing a higher resistance against bactericidal antimicrobials [59-62]. The EPS surface-adherent eDNA binds aminoglycosides, acidifies the system, and helps inducing an on genes instructions based PhoPQ and PmrAB two-component regulatory system. Now an aminoglycoside antibiotic resistant *P. aeruginosa* phenotype can develop [63,64]. In the transition from a planktonic into an interior biofilm lifestyle involved is the messenger molecule c-di-GMP that could have helped strain B8a to metamorphose and to attach after a longer adaptation phase to the HCB crystals and slowly to start with interactions (Figure 1-3, Table 1 & 2); [65]. Metamorphosing concerned *P. aeruginosa* strains possess several advantages. They are able to gain energy by oxygen and nitrate respiration and possess the capability to produce small pyocyanin molecules, phenazines. Pyocyanin is an osmotic pressure cheater and drives extracellular-matrix mediated biofilm expansion. Phenazines can alternate to oxygen act as electron acceptors [66]. With the wealth of microbial energy conserving surviving strategies equipped *P. aeruginosa* strains can survive in water saturated soil pores, sewage sludge flocs, in ocean sediments, and as shown in biofilm structures (Figure 2). In an arable fields to that <sup>14</sup>C labelled HCB was added from the HCB only 1 remaining % could be found after soil extraction and may indicate that bacteria as the *P. aeruginosa* strain B8a may have played a role [11].

The micro scaled chemical gradients in biofilms and the genotypic variation of embedded and micro-clustered bacteria, archaea, fungi, algae in the biofilm interior make a biofilm to a predestined environment for selection, mutation, stochastic gene expression, to an exhibited, on varying conditions adapting environment. A multicellular *In situ* biofilm structure with attached planctomycetes is meanwhile seen as emergent form of bacterial life, a surviving strategy that completely differs from that of free-living bacteria. The laser-based imaging techniques, a variety of sensor types, and other physical, chemical, biological techniques opened a quite comprehensive insight in the biofilm organization, and we understand reasonably how the microbial community inside of a biofilm interacts [67-70]. Apparently nitrate and pyocyanin relieve the biofilm external and internal digestion and all mentioned biofilm properties make biofilms to one of the oldest, ubiquitously dynamic protection system and to the most successful form of life (Figure 2; Table 1) [20]. The biofilm inherent microscale chemical, biological gradients may support an attacking of recalcitrant organic substances as HCB (Table 1) and as the findings of others let assume [30,38]. The oxygen limitation in biofilms to which also *P. aeruginosa* strain B8a was exposed in the described experiment, underlines the finding that such conditions and bacteria exposed to tobramycin, ciprofloxacin, carbenicillin, ceftazidime, chloramphenicol, tetracycline rather tolerate such inhibiting compounds, perhaps also because a gene and phenazine controlled MexGHI-OpmD efflux pump can transport growth inhibiting toxins out of the biofilm [71-75]. Specifically, *Gram-negative*, denitrifying, redox-cycling skilled bacteria as the *P. aeruginosa* strain B8a may in presence of nitrate and supported by self-produced antibiotic active phenazine be enabled to metamorphose from a wild-type into a self-resistant biofilm inhabitant that is then able to attach after a certain adaptation phase on HCB crystals (Figure 2; Table 1).

Biofilm formation at high nutrient availability as conceivable in ocean sediments, in water retaining soil pores, in sewage sludge flocs of wastewater treatment plants, in water films around solid particles can be material destructive [76]. Biofouling, bio-corrosion is the term and the microbial activity beyond is identified as a costly problem for the public but a beneficial one for the biocides, cleaners, and antifouling materials producing industry. Early biofouling warning system are required for saving costly countermeasures, particularly in our industry like organized wastewater treatment plants [77-80]. Because biofouling is today majorly indirectly diagnosed and cured with biocides biofilm formation inside of sewage sludge flocs must be understood, the more because huge amounts of sewage sludge in particularly produced in growing cities with concentrating administrations and industries at WWTPs restricted retention times must be deposited in dumps on arable land on that in soil buffer capacity surpassing amounts industrially produced animal wastes and sewage sludge residues are spread [80-83]. Conceptualized for rural regions are wastewater flooded constructed wetlands in which anaerobic zones are expanding, a biofilm pore, void, channel arrangement develops that favours  $O_2$  respiration,  $NO_3^-$  respiration, and fermentation switching and a co-metabolic attacking of recalcitrant molecules as HCB may start

(Figure 3; Table 1) [84,85].

For minimizing environmental pollution out of overproduced sewage sludge, animal wastes, and plant residues a relatively stable, carbon, nitrogen phosphorus, trace elements, recalcitrant organics, toxins, and other waste components integrating, less volume biochar can be fabricated by the pyrolysis/carbonization technique, but such an approach is only interim solution [66]. The biochar product can be considered as a kind of slow-release fertilizer and may find in agriculture a field of application. Biochar fabrication opens for science and engineering a multidisciplinary study field and contributes to a less alarming storage situation. Into an oligotrophic bulk soil incorporated biochar provides the mostly starving microbial community sufficient adaptation time to start transforming the slowly out of biochar flowing ingredients. As observable in the few millimeters thick rhizo-, drillosphere (earthworm burrow walls) also the out of biochar flowing nutrients may enhance metabolism and a co-metabolizing of pollutants will set in [85-90]. Such a system differs from the situation in wastewater treatment plants where the microbial community is forced in restricted retention times to convert as much as possible from the nutrient rich inflows. Microbes are generally patchy distributed in bulk soils but also in sewage flocs and long-term forced to switch between starving and soil nutrient satiety surpassing soil conditions, e.g., near decaying cadavers (Bruyn 2016 [78]). In the neighborhood of incorporated biochar, it is conceivable that out of biochar diffusing compounds as the plasticizer di-n-butyl phthalate, trichloroethylene, or phenolic structures as HCB are co-attacked, co-metabolized by the mostly hidden working soil microbial capabilities [91]. In this context biochar amendment in agriculture is problem solving when the sewage sludge concerned pyrolysis technique is further developed and reasonably understood [92-99]. A biochar-based agriculture and wastewater treatment integrating approach is a promising interim solution for a mass-production orientated economy which must less trust in a worldwide waste distribution. Understanding the biofilm formation around the HCB crystals better may provide valuable hints towards the hidden working soil microbial world with its mineralizing capabilities of manmade wastes.

## Conclusion

In soils and WWTPs hidden is a high degradation potential of manmade organic compounds. However, longer adaptation times are required for being successful in reducing the incoming recalcitrant organic compound load. In particular recalcitrant organic compounds as HCB afford longer retention times and sewage sludge flocs, soil pores, ocean sediments and biofilms are inspiring environments for recalcitrant organic compounds accumulation and degradation. Favorable seem to be reduced oxygen, not-aerated denitrifying conditions and aerobic, anaerobic interface environment as biofilms. Biochar fabricated out of sewage sludge by the pyrolysis/carbonization technique may provide a worthwhile interim solution for managing in particular WWTPs in enlarging cities and reducing pollution.

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## Compliance with Ethical Standards

### Declaration

I declare that I have no conflict of interest and the using the wastewater concentrate, gained from the wastewater of Civil Engineering Department, University Stuttgart, Germany, was approved at the time of experimentation by the Department Head Prof Hunken.

### Further

I declare that in the submitted study human participants, human data nor human tissue are involved and I state "Not applicable" in this section.

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