Supplemental Data

Bioremediation of polluted air from an industrial plant using rotating biofilter reactor-based technology

Martina Bonadei, Mattia Donà, Alma Balestrazzi, Gabriele Bruni, Daniela Carbonera

Materials and Methods

Isolation of HDB strains

The hydrocarbon-polluted water used for the isolation of the bacteria investigated in the present study was sampled from a wastewater treatment plant located in Lombardy (Italy). Aliquots (5 mL) of hydrocarbon-polluted water were mixed with 100 mL of sterile dH_2O into a bottle. After 30 min of shaking on a roll-bed, aliquots (0.01 mL) were plated in replicates on the solid media LB (Luria Bertani) medium (Oxoid, Italy; Trypton 10 g L^{-1}, Yeast Extract 5 g L^{-1}, NaCl 10 g L^{-1}) and NB (Nutrient Broth) medium (Oxoid, Italy; Beef Extract 3 g L^{-1}, Peptone 5 g L^{-1}, agar 15 g L^{-1}, pH 7.0). Plates were then incubated for three days at 37°C. This step was repeated at least three times. For each bacterial isolate, single colonies were obtained using the pour-plate technique (Dubey & Maheshwari, 2005). Each selected colony was inoculated into a flask containing the NCM (Non-Carbon Source) medium (NaCl, 2.0 g L^{-1}; $MgCl_2.6H_2O$, 0.4 g L^{-1}; $CaCl_2.2H_2O$, 0.1 g L^{-1}; Na_2SO_4, 4.0 g L^{-1}; NH_4Cl, 0.25 g L^{-1}; KH2PO4, 0.2 g L^{-1}; KCl, 0.5 g L^{-1}; selenite solution-$NaSeO_3$, 1.0 mL L^{-1}) supplemented with increasing benzene concentrations (0.005 µg mL^{-1}; 0.01 µg mL^{-1} ; 0.015 µg mL^{-1}). The isolates able to survive to benzene exposure were collected and stored for subsequent characterisation. For each isolate, a single colony was grown o.n. at 37°C in 3 mL of liquid LB medium under continuous shaking. The resulting culture was subsequently used to inoculate a flask containing 500 mL of LB medium which was incubated at 37°C for 24 h. Bacterial growth was monitored by measuring the optical density OD_{600nm} with a V-530 spectrophotometer (Jasco Europe S.r.l., Italy). For the swarming assay, a single colony of HDB1, HDB2 and of the consortium was cultured o.n. at 37°C in 3 mL of liquid LB medium under continuous shaking. A 2 µl droplet for each isolate was inoculated on the center of plates containing LB medium at 1.5% and 0.7% agar, respectively, and after 24 h of incubation at 37°C, the distance covered by the swarming bacteria from the inoculation point was measured

Identification and phylogenetic affiliation

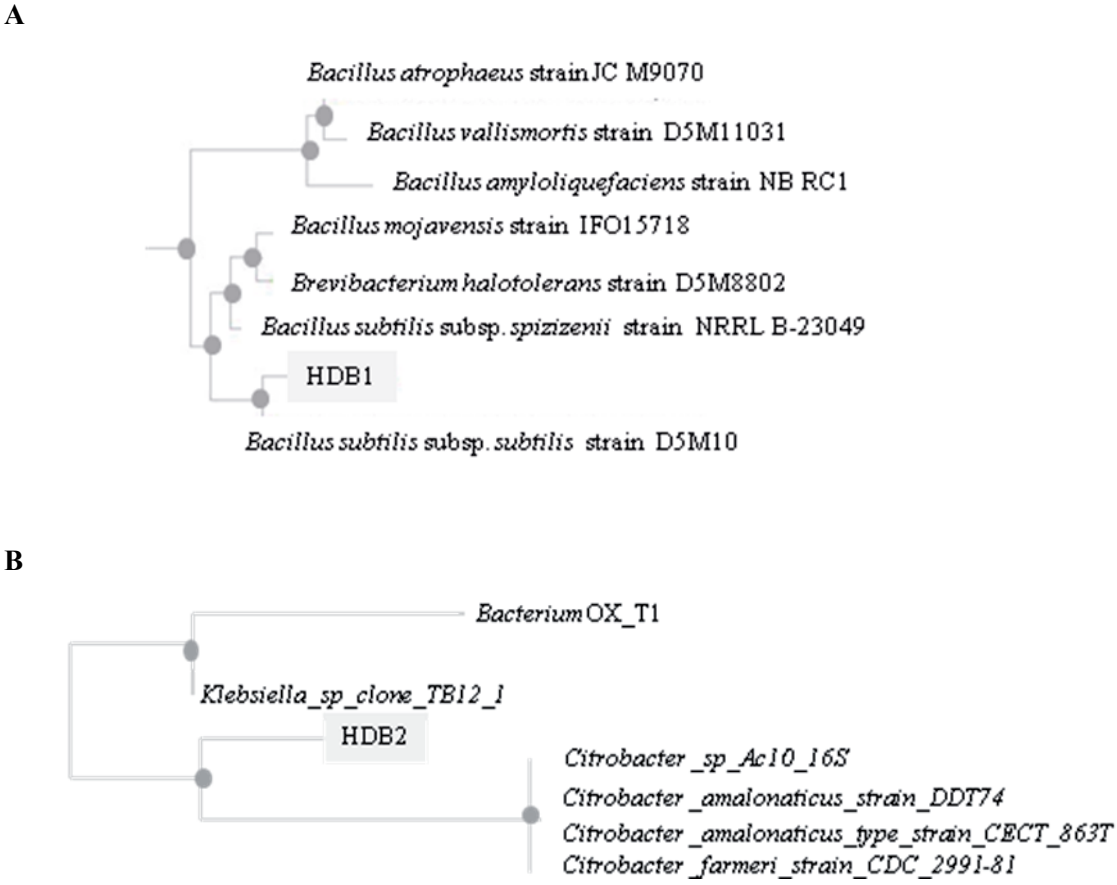
Amplification of 16S rDNA sequences was carried out using purified DNA (15-30 ng) and the universal bacterial oligonucleotide primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCANCCRCA-3') at the following conditions: 94°C for 1 min, 55°C for 1 min, 72°C for 3 min (35 cycles) (Suzuki and Giovannoni, 1996). PCR reactions were performed in a final volume of 30 µL containing 0.2 mM premixed deoxynucleoside triphosphates (dNTPs) (M-Medical S.r.l**.,** Italy), 1.5 mM $MgCl_2$ and 2.5 U *Taq* DNA Polymerase (DyNAzyme II, Italy), using a T Gradient apparatus (Biometra GmbH, Germany). PCR products were separated on 1.0% (w/v) agarose gels (Duchefa Biochemie B.V, The Netherlands) and purified using the GFX^{TM} PCR DNA and Gel Band Purification kit (Amersham Italia, Italy). Sequence analysis was performed at Parco Tecnologico Padano (Lodi, Italy). Construction of the phylogenetic tree was performed using the FAST Minimum Evolution Method (FastME, http://www.ncbi.nlm.nih.gov) (Desper and Gascuel, 2004) and Dendroscope 3 (http://www.dendroscope.org) (Huson et al., 2007).

Results

Molecular taxonomy of HDB1 and HDB2 isolates

As a first step, a preliminary identification of the bacterial strain contained in the 'Biowheel 2.0' biofilter was carried out. The HDB1 isolate (GenBank accession number KF801671) sampled in hydrocarbon-polluted wastewater has been identified as *Bacillus* spp. while the HDB2 isolate (GenBank accession number JQ317684), obtained by sampling from 'Biowheel 2.0' during a preliminary investigation, has been classified as *Citrobacter amalonaticus,* (Supplemental Fig. S1). A bacterial consortium, obtained by mixing the pure cultures of the

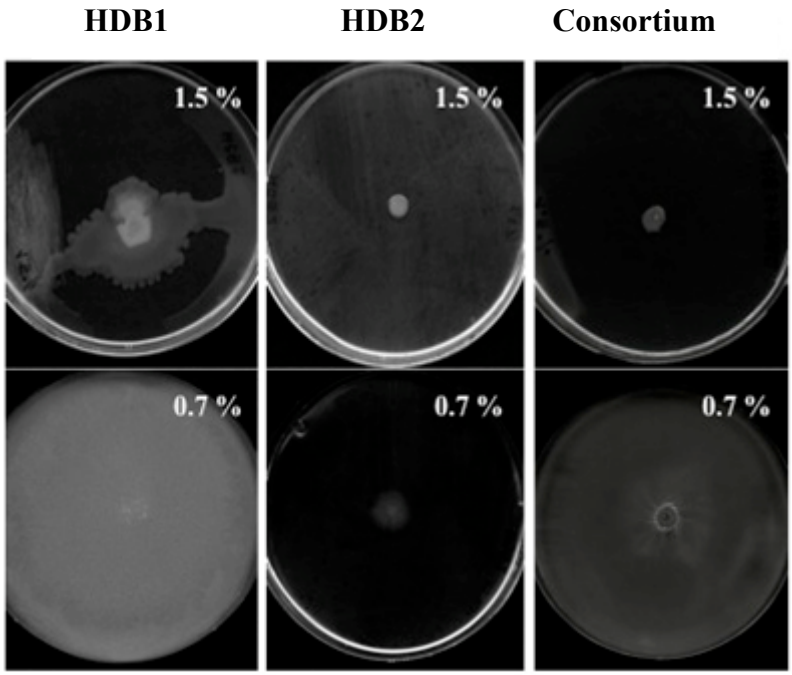
Bacillus spp. and *C. amalonaticus* isolates, was subsequently used to inoculate 'Biowheel 2.0' in order to assess the ability of these bacteria in removing benzene and NMVOCs from polluted gaseous flow samples.



Supplemental Fig. S1. Phylogram obtained based on the phylogenetic analysis of 16S rDNA sequence of HDB1 (A) and HDB2 (B) isolates.

Swarming ability

The HDB2 isolate highlighted a barely detectable swarming ability, with limited colony expansion in the case of both 1.5% and 0.7% agar plates, with a putative swarming rate of 0.4 mm h^{-1}. The HDB1-HDB2 consortium showed a hybrid swarming behavior, with a limited colony expansion when inoculated on 1.5% agar plates, and a full coverage of the plate surface in the case of 0.7% agar plates, with clear bacterial migration tracks and signs of swarming 'waves' (Supplemental Fig. S2), due to a non-excessive thickness of the layer of bacterial culture. The swarming rate of the consortium was estimated in 5.1 mm h^{-1}.



Supplemental Fig. S2. Swarming rate of strain HDB1, HDB2 and the consortium after 24 h of incubation of 1.5% and 0.7% agar-containing LB plates.

Description of 'mold-casting' process and gaseous flows at the industrial plant

Levels of benzene and NMVOCs were quantified in different classes of gaseous flow found in iron and steel industrial plants: *i)* 'cold box', *ii)* 'in shell' and *iii)* 'mix'. 'Mold-casting' relies on the use of temporary, non-reusable molds for the industrial-scale production of a wide range of specific components (Li et al., 2013). The three different processes are routinely used at the industrial plant hereby investigated, frequently within the same working day. 'Biowheel 2.0' was transferred at the industrial plant and tested in the absence of bacterial inoculum. The industrial process, including gaseous flow sampling and biofilter activity is represented in Fig. S3. The polluted gaseous flow entering the non-inoculated rotating biofilter, defined as Non Treated (NT) inlet flow, was used as a control in comparison with the inlet gaseous flows exposed to the HDB1-HDB2 consortium. Evaluation of NT inlet flow allowed testing the hydraulic capacity of the apparatus, based on the features of the inlet gaseous flow. The HDB1-HDB2 consortium inoculated into the rotating biofilter 'Biowheel 2.0' required three weeks to adapt and develop optimal growth and biomass conditions.

The gaseous flows released during the industrial process were immediately directed towards the apparatus (Fig. S3), where they reached the 'Biowheel 2.0' inoculated with bacteria. For each gaseous flow analyzed, sampling was carried out at three different time points. The starting point for the experiment was the time point (T_0) when 50% of the volume of the process tunnel was filled in with the industrial material (Fig. S3). Sampling was then performed at 30, 60 and 90 min (T_{30}, T_{60} and T_{90}) following T_0. Sampling was replicated three times every two weeks. Benzene and NMVOCs levels measured in the inlet flow delivered to 'Biowheel 2.0' in the absence (NT) and presence of HDB1-HDB2 consortium is provided in Supplemental Data (see Supplemental Table S1). The observed fluctuations in benzene and NMVOCs indicate that organic pollutants concentration can vary significantly within the process tunnel during standard production. Odor impact was evaluated in the inlet gaseous flows with the force choice method (Supplemental Table S2). Considering the observed fluctuations in the level of pollutants emitted at the industrial site, an effective bioremediation system would require microorganisms

able to quickly adapt their metabolism to the significant changes in the amount of hydrocarbons. The occurrence of sudden fluctuations in pollutant concentration within the inlet flow provides useful informations on changes in bioremediation performance, since an active biofilter should be able to handle these events to maintain the highest removal efficiency (Rene et al., 2005).

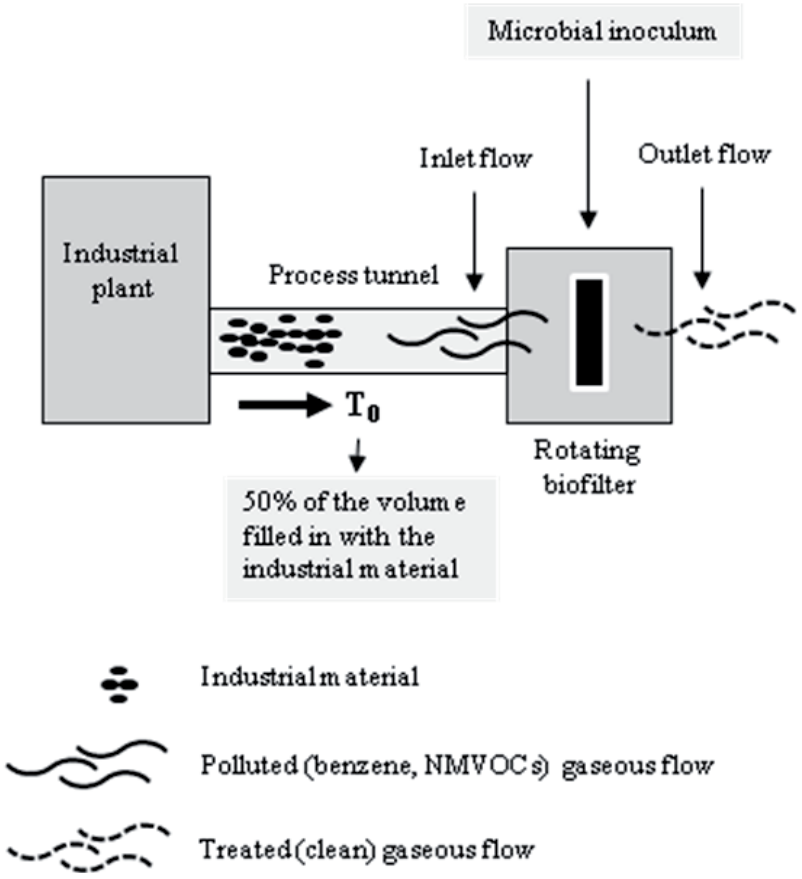


Fig. S3. 'Biowheel 2.0' was transferred at the industrial plant and connected with the process tunnel. The polluted gaseous flows released during the industrial process were immediately directed towards the apparatus. The different inlet gaseous flows ('in shell', 'cold box' and 'mix') reached 'Biowheel 2.0' inoculated with bacteria. The starting point for the experiment was the time point (T_0) when 50% of the volume of the process tunnel was filled in with the industrial material. Sampling was then performed at 30, 60 and 90 min following T_0. NMVOCs, non-methane volatile compounds.

Benzene and NMVOCs content and associated odor impact in the inlet gaseous flows

As shown in Supplemental Table S1, the amount of benzene measured in the inlet flow delivered to the rotating biofilter 'Biowheel 2.0' in the absence of microbial inoculum (Untreated control, NT) significantly (P = 0.0102 and P = 0.0066, respectively) varied during the sampling time from 6.30 ±1.26 mg m^{-3} (T_{30}) and 6.70 ±1.34 mg m^{-3} (T_{60}) when comparing to T_{90} (2.40 ±1.48 mg m^{-3}). Significant (P =0.0131) fluctuations were also observed in the level of NMVOCs contained in the different NT samples, namely 29.50 ±5.90 mg m^{-3} (T_{30}), 34.20 ±6.84 mg m^{-3} (T_{60}) and 18.20 ±3.64 mg m^{-3} (T_{90}). The reported values refer to 'in shell' gaseous flow. The observed fluctuations indicate that the amount of organic pollutants may vary significantly within the process tunnel during the standard production activity. Subsequently, the benzene and NMVOCs concentrations were measured in the different inlet gaseous flows ('in shell', 'cold box' and 'mix') delivered into the rotating biofilter 'Biowheel 2.0' inoculated with bacteria (Supplemental Table S1). Non-significant fluctuations (P = 0.120) were detected. As for the 'in shell' gaseous flow, benzene levels ranged from 5.90 ± 1.18 mg m^{-3} (T_{30} and T_{60}) to 8.20 ± 1.64 mg m^{-3} (T_{90}). The estimated amount of NMVOCs in the same gaseous flow at the different sampling times was 16.70 ± 3.34 mg m^{-3} (T_{30}), 23.50 ± 4.70 mg m^{-3} (T_{60}) and 17.00 ± 3.40 mg m^{-3} (T_{90}). When the inlet flow resulting from the 'cold box' process was analysed, the benzene concentration was 10.60 ± 2.12 mg m^{-3} (T_{30}), 8.20 ± 1.64 mg m^{-3} (T_{60}) and 12.50 ± 2.50 mg m^{-3} (T_{90}). Otherwise, significant fluctuations (P = 0.017 and P = 0.025, respectively) were measured in the level of NMVOCs at T_{30} and T_{60} (30.40 ± 7.30 mg m^{-3} and 34.50 ± 8.20 mg m^{-3}) when compared to T_{90} (10.00 ± 2.50 mg m^{-3}) (Supplemental Table S1). Finally, when considering the 'mix' gaseous flow, the estimated benzene contamination was 12.20 ± 2.44 mg m^{-3} (T_{30}), 10.60 ± 2.12 mg m^{-3} (T_{60}) and 9.10 ± 1.82 mg m^{-3} (T_{90}) while the NMVOCs were found at 41.60 ± 9.90 mg m^{-3} (T_{30}), 55.00 ± 13.00 mg m^{-3} (T_{60}) and 45.50.10 ± 10.90 mg m^{-3} (T_{90}) (Supplemental Table S1).

Supplemental Table S1. Benzene and NMVOCs (Non-Methane Volatile Organic Compounds) concentrations measured in the different inlet gaseous flows ('in shell', 'cold box', and 'mix') released by the industrial process and collected at the industrial plant. The gaseous flows were delivered into the rotating biofilter 'Biowheel 2.0' for bioremediation. For each gaseous stream typology analysed, sampling was carried out at three different time points. The starting point for the experiment was the time point (T_0) when 50% of the volume of the process tunnel was filled in with the industrial material. Sampling was then performed at 30, 60 and 90 min (T_{30}, T_{60}, T_{90}) following T_0.

		GASEOUS FLOWS		
		+ Bacterial inoculum		
	NT (*)	'in shell'	'cold box'	'mix'
Benzene (mg m^{-3})	**T_{30}** 6.30 ± 1.26	5.90 ± 1.18	10.60 ± 2.12	12.20 ± 2.44
	T_{60} 6.70 ± 1.34	8.20 ± 1.64	8.20 ± 1.64	10.60 ± 2.12
	T_{90} 2.40 ± 1.48	5.90 ± 1.18	12.50 ± 2.50	9.10 ± 1.82
NMVOCs (mg m^{-3})	**T_{30}** 29.50 ± 5.90	16.70 ± 3.34	30.40 ± 7.30	41.60 ± 9.90
	T_{60} 34.20 ± 6.84	23.50 ± 4.70	34.50 ± 8.20	55.00 ± 13.00
	T_{90} 18.20 ± 3.64	17.00 ± 3.40	42.00 ±10.00	45.50 ± 10.90

(*) Untreated control: inlet flow delivered to the biofilter in the absence of microbial inoculum

Odor impact of the inlet gaseous flows

The odor impact (Olfactometric Units; O.U.) was evaluated in the inlet gaseous flows with the force choice method. The reported values refer to the starting point for the experiment, or the time point (T_0) when 50% of the volume of the process tunnel was filled in with the industrial material. Results are summarized in Supplemental Table S2. The analysis was carried out in the different gaseous flows, previously described, 'cold box', 'in shell' and 'mix'. The polluted gaseous flows entering the non-inoculated rotating biofilter 'Biowheel 2.0', defined as Non Treated (NT) inlet flow, was used as a control in comparison with the treated inlet gaseous flows exposed to the bacterial consortium.

As shown in Supplemental Table 2, the average O.U. measured in the inlet flow delivered to the biofilter 'Biowheel 2.0' in the absence of microbial inoculum (Untreated control, NT) was

1630 ± 489 O.U. m^{-3}. As for the 'in shell' gaseous flow, the estimated amount was 2400 ± 720 O.U. m^{-3}. The same values were recorded for 'cold box' and 'mix' gaseous flows, namely 3800 ± 980 O.U. m^{-3} (Supplemental Table S2).

Supplemental Table S2. Average Olfactometric Units (O.U.) measured in the different inlet gaseous flows ('in shell', 'cold box', and 'mix') collected at the industrial plant and delivered into the rotating biofilter Biowheel 2.0' for bioremediation. The reported values were measured at T_0, the starting point for the experiment was the time point (T_0) when 50% of the volume of the process tunnel was filled in with the industrial material.

	GASEOUS FLOWS			
		+ Bacterial inoculum		
	NT (*)	'in shell'	'cold box'	'mix'
O. U. m^{-3}	1630 ± 489	2400 ± 720	3800 ± 980	3800 ± 980

(*) Untreated control: inlet flow delivered to the biofilter 'Biowheel 2.0' in the absence of microbial inoculum

Odor sampling and analysis by panel test method and electronic-nose technology PEN3

Odor concentration in the gaseous flow samples was measured by dynamic olfactometry. The tests were carried out inside an odor-free, clean laboratory with selected and trained panelists. Each samples was diluted in the TO-7 Olfactometer (Ecoma GmbH, Germany) several times differing from each other by a factor of two and presented to the panelists three times. Dilutions were made using odor-free air supplied by a compressor fitted with carbon filters and an air dryer. The olfactometer is a computer controlled semi-automatic instrument with four panel member places and computes the odor concentration by means of a special computer program based on the perception response data of panelists. This method employ a 'yes/no' technique and determines how many times a sample must be diluted with odor-free air to be at the threshold of detection by 50% of the panel. The number of required dilution defines the odor concentration as olfactometric units (O.U.) m^{-3}. Samples of inlet and outlet gaseous

flows, treated with the rotating biofilter 'Biowheel 2.0' and untreated controls, were also analyzed using the Portable Electronic Nose PEN3 (AIRSENSE Analytics GmbH, Germany) which simulates the mental process of recognition and memorization of the human olfactory system. Similarly to the human electric receptors which send messages to the brain, the PEN3 Metal Oxide Sensors, that have been treated with several layers of metal and appropriate temperatures to become sensitive to different NMVOCs classes, are able to send electric signals to the computer. Each PEN3 sensor is programmed with a specific working temperature (50-100°C), and this feature enables to expand the range of detection/measurement of NMVOCs perceived by the instrument. Data obtained from measurements are referred to the percentage of removal efficiency (RE), as the capacity of the system to modify the inlet flow in order to obtain an odorless air.

Supplemental Table S3. Data revealed by electron nose technology. These results are referred to the gaseous flow measured in the different process typologies ('in shell', 'cold box', and 'mix'). Data obtained from measurements are referred to the percentage of Removal Efficiency (RE), as the capacity of the system to modify the inlet flow in order to obtain an odorless air.

Gaseous flow	**Removal Efficiency* (%)**
'in shell'	84.31
'mix'	83.12
'cold box'	85.40

* measuring method: 10 sens. MOS-PEN3; calculation method: Partial Least Square Regression

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