

## Bacterial Dynamics of Biofilm Development During Toluene Degradation by *Burkholderia vietnamiensis* G4 in a Gas Phase Membrane Bioreactor

Kumar, Amit<sup>1</sup>, Jo Dewulf<sup>1</sup>, Tom Van De Wiele<sup>2</sup>, and Herman Van Langenhove<sup>1\*</sup>

<sup>1</sup>Research Group of EnVOC, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

<sup>2</sup>Laboratory Microbial Ecology and Technology (LabMET), Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

Received: November 21, 2008 / Accepted: March 4, 2009

**In this study, the dynamics of living cells (LC) and dead cells (DC) in a laboratory-scale biofilm membrane bioreactor for waste gas treatment was examined. Toluene was used as a model pollutant. The bacterial cells were enumerated as fluoromicroscopic counts during a 140 operating day period using BacLight nucleic acid staining in combination with epifluorescence and confocal laser scanning microscopy (CSLM). Overall, five different phases could be distinguished during the biofilm development: (A) cell attachment, (B) pollutant limitation, (C) biofilm establishment and colonization, (D) colonized biofilm, and (E) biofilm erosion. The bioreactor was operated under different conditions by applying different pollutant concentrations. An optimum toluene removal of 89% was observed at a loading rate of 14.4 kg m<sup>-3</sup> d<sup>-1</sup>. A direct correlation between the biodegradation rate of the reactor and the dynamics of biofilm development could be demonstrated. This study shows the first description of biofilm development during gaseous toluene degradation in MBR.**

**Keywords:** Biodegradation, membrane bioreactor, cell viability, microbial biofilm, toluene, CLSM, VOC

Biological treatment of waste gases has advantages compared with physical–chemical treatments when volatile organic compounds (VOCs) are biodegradable and their concentrations are low. Biofilters (BF) and biotrickling filters (BTF) are the most frequently used biological techniques for waste gas treatment. In both techniques, gas flows through a packed bed of carrier material on which microorganisms grow as a biofilm [21]. The biofilm is covered by a water layer, forming a barrier for hydrophobic compounds between

the microorganisms and the waste gas [24]. The membrane bioreactor for waste gas treatment (MBRWG) is a technological advantage that makes it possible to separate gas and liquid phases. Pollutants diffuse through the membrane and are subsequently degraded by the microorganisms in the biofilm. However, the success of this biotechnology is sometimes hampered by the inactivation of the microbial community and the clogging due to an excess of biomass [9, 19].

MBRWG for toluene removal has often been studied with respect to process optimization and performance [11]. However, little is known about the biofilms on the membrane surfaces [19], and up till now the biofilm is considered as a “black box”.

In addition, the biofilm structure is of special importance in the operation of biofilm reactors [22]. Biofilms in bioreactor systems are highly organized structures of cellular aggregates embedded in the extracellular polymer substances (EPS) [5]. Their structure includes voids and channels that increase the influx of substrates and nutrients to the inner parts of the biofilm and facilitate the efflux of wastes [23]. To date, no information exists about the biofilm structure and dynamics in MBRWG. In bioreactors, the living cells (LC) within the biofilms are usually quantified by plating techniques based on colony forming units. However, this method may enumerate only a minority of the microbial populations as it does not take into account the viable but not culturable (VBNC) microorganisms [14]. In addition, two studies conducted on a monoculture of *Pseudomonas putida* demonstrated that pollutants could decrease the cell culturability on selective media [15, 16]. In order to identify microorganisms and to determine their quantity in biofilms without cultivation, culture-independent methods such as the fluorescence *in situ* hybridization (FISH) method were developed [1]. To date, no information exists about the biofilm structure and dynamics in MBRWG.

Owing to the above-mentioned complexity, current understanding of the biofilm systems for waste gas treatment

\*Corresponding author

Phone: +3292645953; Fax: +3292646243;  
E-mail: herman.vanlangenhove@ugent.be  
Web link: www.envoc.ugent.be

is limited. In fact, very few examinations of the bacterial dynamics during different operating conditions and long-term operation under transient conditions of such systems have been carried out. Therefore, the aim of the present study was to monitor biofilm dynamics by running a membrane bioreactor under different operational conditions challenged with toluene as a model pollutant.

## MATERIAL AND METHODS

### Bioreactor Set-Up

Commercially available polydimethylsiloxane (PDMS)/polyacrylonitrile (PAN) composite membrane (GKSS, Germany; 40 cm<sup>2</sup> effective membrane area) was used, consisting of PDMS as the hydrophobic dense top layer with a thickness of 0.3 μm and PAN as a support layer material with a thickness of 185 μm. The membrane was incorporated into a Perspex reactor module. A pure microbial culture of *Burkholderia vietnamiensis* G4 was selected on the basis of literature data for toluene biodegradation and was obtained from BCCM/LMG Laboratory of Microbiology, Ghent University, Belgium. The mineral medium (MM) used for the MBR consisted of 1 g/l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 1 g/l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 1 g/l<sup>-1</sup> KNO<sub>3</sub>, 1 g/l<sup>-1</sup> NaCl, 0.2 g/l<sup>-1</sup> MgSO<sub>4</sub>, 26 mg/l<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 5.2 mg/l<sup>-1</sup> EDTA-Na<sub>4</sub>(H<sub>2</sub>O)<sub>2</sub>, 1.5 mg/l<sup>-1</sup> FeCl<sub>2</sub>·4H<sub>2</sub>O, 0.1 mg/l<sup>-1</sup> MnCl<sub>2</sub>·2H<sub>2</sub>O, 0.012 mg/l<sup>-1</sup> CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.07 mg/l<sup>-1</sup> ZnCl<sub>2</sub>, 0.06 mg/l<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 0.025 mg/l<sup>-1</sup> NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.025 mg/l<sup>-1</sup> NaMo<sub>4</sub>·2H<sub>2</sub>O, 0.015 mg/l<sup>-1</sup> CuCl<sub>2</sub>·2H<sub>2</sub>O. For all the experiments described herein, the MBR was rinsed with ethanol, and the mineral medium and heat-resistant reactor parts were autoclaved prior to the experiments. The experimental set-up used in this study has been previously described [10].

### Reactor Operation

The reactor was operated for a period of 140 days after seeding with *Burkholderia vietnamiensis* G4, which had been grown in a mineral medium with toluene as a sole carbon and energy source. The MBRWG was subjected to different load conditions to determine the removal characteristics through the unit. The toluene inlet concentrations (C<sub>in</sub>) were changed between 0.8 to 4.0 g/m<sup>3</sup>. The mass loading rate (LR) was 3.6 to 15.5 kg m<sup>-3</sup> d<sup>-1</sup>. The performance of the membrane bioreactor was evaluated by removal efficiency.

### Analytical Methods

Gas phase toluene concentration was measured using a Varian 3700 gas chromatograph (Varian Associates, Inc.) coupled to an FID detector. Gas samples were taken in triplicate with a 1-ml Vici gas syringe. Toluene removal efficiencies were determined on a daily basis. Different loadings were applied and the removal efficiency was measured at each loading. Water phase toluene concentrations were determined by taking 1-ml water samples with a plastic syringe (BD plastipak). The samples were brought into a 4.5-ml vial with a Teflon lined Mininert screw cap and placed in a thermostatic bath at 30.0°C. After 2 h, 1 ml of the gas phase was sampled and injected into the gas chromatograph. Biofilm samples were periodically collected from the bioreactor and analyzed by CSLM and bacterial enumeration. For bacterial enumeration, two different methods were used: (1) agar plates to count the number of live and dead cells, and (2) CSLM. Cell dry weight was determined gravimetrically [2].

### Confocal Laser Scanning Microscopy (CLSM)

The bacterial LC and DC that developed in the biofilm of a toluene-degrading MBR were enumerated as fluoromicroscopic counts using nucleic acid staining and a direct epifluorescence filter technique. For staining of biofilms, a biofilm area of 0.5–1.0 cm<sup>2</sup> was taken from the membrane and mounted in a small Petri dish. The live/dead BacLight Bacterial Viability Kit L-13152 (Molecular Probes) was used for staining according to the manufacturer's instructions and imaged with a Leica SP5 AOBS confocal microscope (HC PL APO 20.0×0.70 IMM UV), using the 543 nm HeNe laser line for the propidium iodide (PI). SYTO 9 was excited with the 488 nm line of a Multi Argon laser. Sequential scanning was performed, with an emission bandwidth 493–590 nm for SYTO9 and an emission bandwidth of 625–710 nm for PI.

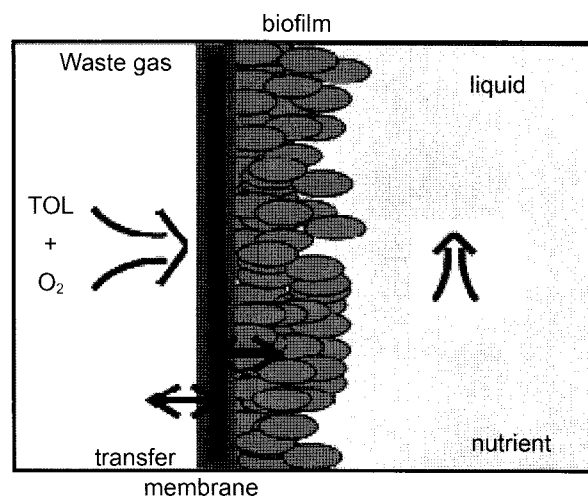
### Three-Dimensional (3D) Visualization

Digital image analysis of the 3D data set was done as follows: Images were visualized by using the microscopic software (Leica) for maximum intensity projection. The surface isosurface was used to create biofilm 3D visualizations; in this approach, 3D surfaces were created to encase the biomass by interconnecting its boundary voxels by using Amira 5.2 software. Therefore, biofilm visualizations created in this manner were a geometric representation of a surface (termed as isosurface) from a 3D volume data set. CSLM z-stacks were processed by re sampling and segmenting the images according to a threshold that was selected according to a fluorescence intensity histogram of the tagged image file format (TIFF) files. In this manner, segmentation partitioned the images into background and biomass voxels, and this was further user verified by manually comparing segmented biomass with its 2D original.

## RESULTS AND DISCUSSION

### MBR Performance and Different Phases of Developed Biofilm

In this MBR, toluene-loaded air diffuses through the porous side of the membrane and is subsequently degraded



**Fig. 1.** Principle of gaseous toluene biodegradation in the membrane bioreactor.

by the microorganisms in the biofilm attached to the dense membrane. After 2 days, >60% toluene removal was observed. The microbial suspension was replaced by fresh MM manually, and thus all non-adhering cells were removed. On day 10 (phase A), the gas residence time ( $\tau$ ) was set at 11 s. Toluene removal efficiency was 65% with a LR of  $6.9 \text{ kg m}^{-3} \text{ d}^{-1}$ . On day 30 (phase B), a removal efficiency of 45% was observed and was in the same order during the next 36–43 days. This may be due to the mechanical problems (leakage at the liquid side and shutdown of peristaltic pump) in the reactor set-up. This probably caused depletion of nutrient supply for the biofilm and lower reactor performance. However, on day 44, by increasing the residence time to 28 s, consequently decreasing the average loading rate to  $1.02 \text{ kg m}^{-3} \text{ d}^{-1}$ , a removal efficiency of 99% was observed. This period (0–44 days) was considered as a reactor start-up.

After membrane bioreactor start-up different phases (C to E) were considered: phase C (day 60), phase D (day 80), and phase E (day 140). During each of these phases, the MBRWG was subjected to different load conditions to determine the removal characteristics through the unit. The toluene inlet concentrations ( $C_{in}$ ) were changed between 0.8 and  $4.0 \text{ g/m}^3$ . The loading was kept constant until the removal efficiency reached steady state to be sure that the reactor performance was stable over time at a given loading rate. The mass loading rate (LR) was increased from 3.6 to  $15.5 \text{ kg m}^{-3} \text{ d}^{-1}$ . On day 60, at a LR of  $3.6 \text{ kg m}^{-3} \text{ d}^{-1}$  ( $\tau=24 \text{ s}$ ), a removal efficiency of 98% was observed. On day 80, at a LR of  $14.4 \text{ kg m}^{-3} \text{ d}^{-1}$  ( $\tau=24 \text{ s}$ ), removal efficiency was 89%. On day 140, at a LR of  $15.5 \text{ kg m}^{-3} \text{ d}^{-1}$  ( $\tau=10 \text{ s}$ ), removal efficiency decreased to 78%. An overview of the results is plotted in Fig. 2 and demonstrates that the removal efficiency depends on both the gas residence time and the loading rates. The lower removal efficiency obtained at decreasing gas residence time can be explained by

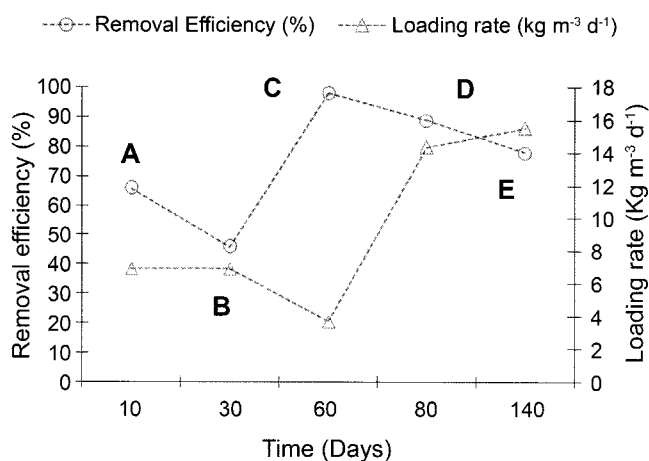


Fig. 2. Effect of loading rate on the toluene removal efficiency and different phases of developed biofilm.

change to a mass transfer rate controlled biosystem. The flux of toluene into the biofilm declines at decreasing residence time, due to lower inlet concentration gradients over the membrane surface.

### Dry Weight and Thickness of Biofilm

The biofilm growth normalized as biomass dry weight could not be directly compared with the growth of a suspended bacterial culture because of (i) the constant movement of microorganisms between the biofilm and the liquid phase (*e.g.*, bacteria release, biofilm sloughing), (ii) the different physiological state of bacteria in the biofilm as compared with the planktonic counterparts, and (iii) the substrate limitation because of the decrease of mass transfer in the biofilm [5]. The thickness and dry weight of the biofilm were assessed as a function of time (Fig. 3). Kirchner *et al.* [8] reported that the biologically active layer of the biofilm was limited to a depth of  $100 \mu\text{m}$  because of a diffusion-controlled nutrient and oxygen supply. In another study, the first indication of nutrient and oxygen shortage was found at a biofilm thickness of  $25 \mu\text{m}$  [20]. This was done to assess the biofilm growth activity over a time period.

### Enumeration of Living and Dead Cells During Toluene Biodegradation

The LC and DC within the biofilm developed on the membrane in a toluene-degrading MBR were enumerated as fluoromicroscopic counts during the 140 operating days period using nucleic acid staining and direct epifluorescence filter technique. The biofilm developed could be differentiated into different phases: (A) cell attachment, (B) pollutant limitation, (C) biofilm establishment and colonization, (D) colonized biofilm, and (E) biofilm erosion. The biofilm stained with live (green)/dead (red) is represented as maximum-intensity projections in Fig. 4. A three-phase development is a common conceptual model in naturally occurring biofilms [4], and it has also been described in pollutant-degrading biofilms [17].

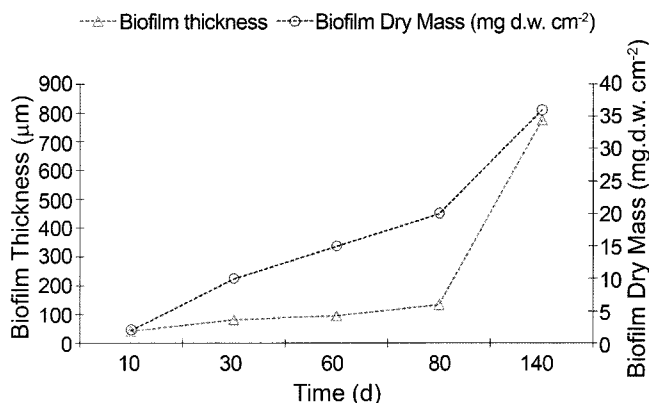
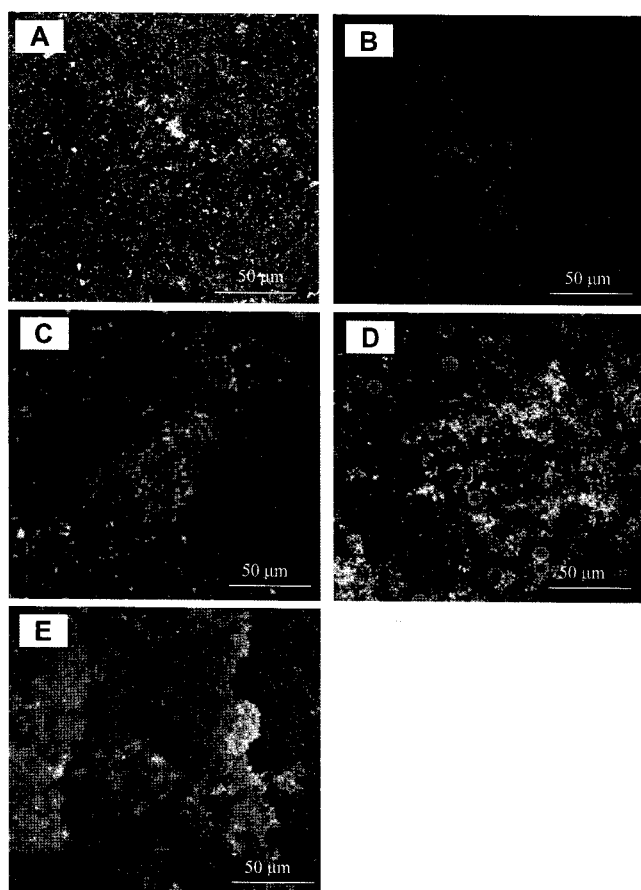


Fig. 3. Biofilm thickness and dry weight as a function of time.

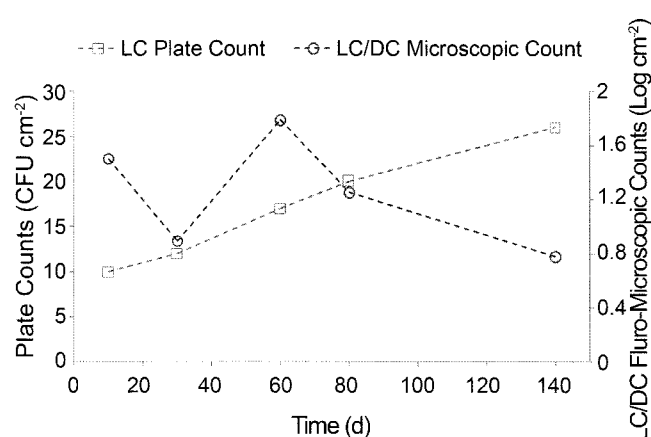


**Fig. 4.** Biofilm development after 10, 30, 60, 80, and 140 days (A, B, C, D, and E, respectively) during toluene biodegradation by *Burkholderia vietnamiensis* G4 in a membrane bioreactor. The biofilm stained with live (green)/dead (red) is represented as maximum-intensity projections.

### Comparison of Two Methods to Enumerate Bacterial Cells

The microbial LC and DC within the biofilm developed in MBR were enumerated using two nucleic acid-staining fluorochromes (SYTO9 and propidium iodide). As the efficacy of these fluorochromes to stain LC and DC was verified on the microbial community used for toluene removal in the reactor, the red fluorescing cells were considered as dead and green fluorescing cells as alive in this study. The kit has already been used to distinguish living bacteria from dead bacteria isolated from different habitats [3, 6, 13, 18]. Bacterial LCs in the biofilm were enumerated according to two different techniques: a culture-dependent (plate counts) and a culture-independent technique (fluoroscopic counts) (Fig. 5). The results obtained with the latter direct count technique were first compared with the number of LCs obtained by plate counts. As expected, the plate count technique allowed the enumeration of less cells than the direct count technique.

An explanation is that the loss of culturability could be linked to pollutant-induced stress. In a study with pure

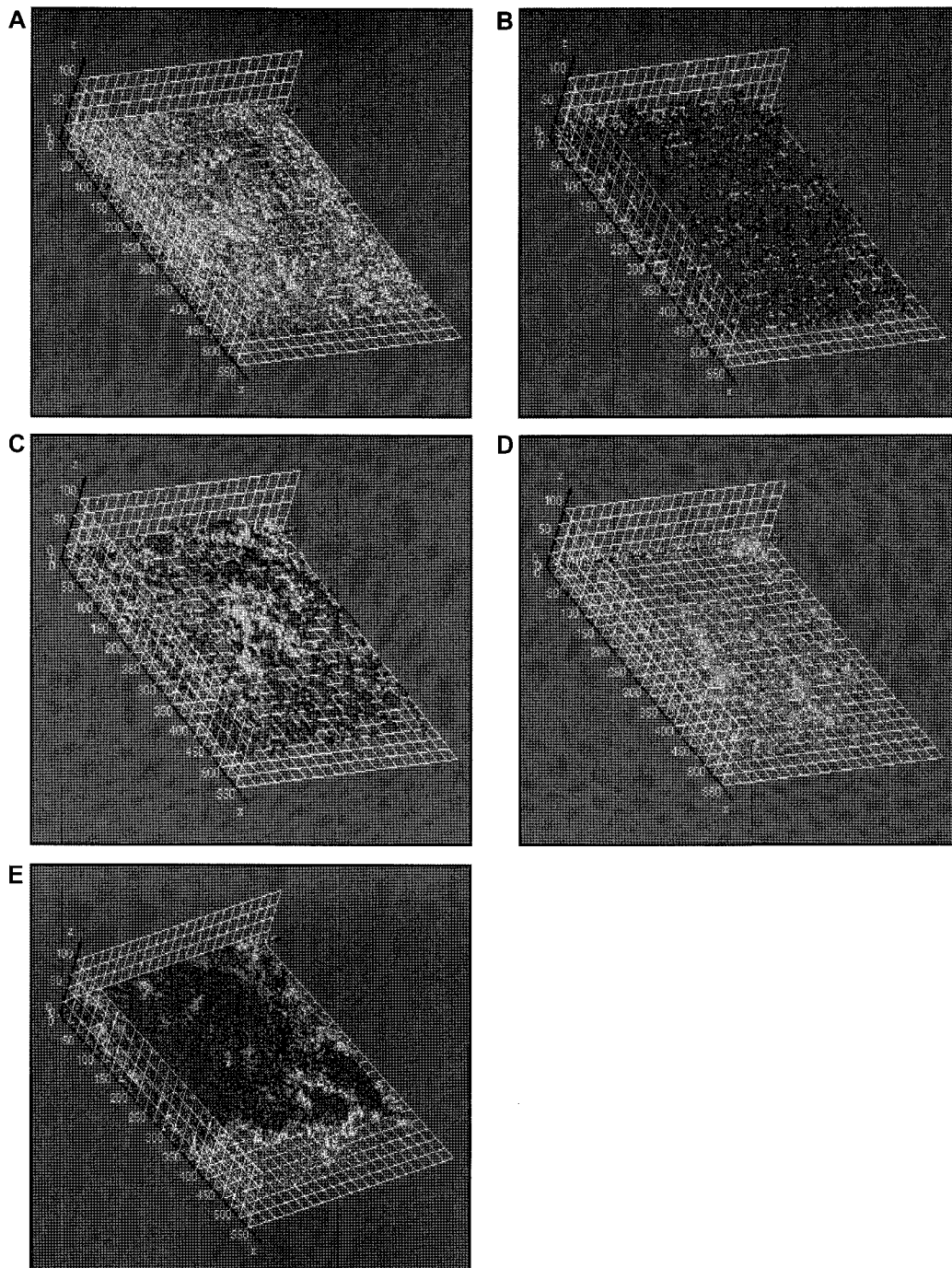


**Fig. 5.** Living cell (LC) and dead cell (DC) dynamics within the developed biofilm (LC determined by plating count method, and LC/DC determined by fluoroscopic count method).

cultures of *P. putida*, Jones *et al.* [7] showed that the fraction of LCs that cannot grow on a selective medium increased slowly over the duration of toluene exposure in a vapor phase bioreactor. In the same bioprocess, an in-depth physiological analysis of the *P. putida* biofilm also revealed that the total respiring cell fraction decreased as the inlet toluene concentration increased [25]. It is therefore possible that, in this study, part of the bacterial LCs in the biofilm lost their culturability after long-term exposure to toluene. The present study revealed that the LCs were dominant in the biofilm during phases C and D compared with the others. The irregular shape (phase E) of the biofilm could be due to the accumulation of inactive organic matter. This organic matter could either come from extracellular polymers or cellular products of leakage and bacterial lysis [12]. The constant accumulation of such organic matter could therefore be one of the key factors responsible for the eventual clogging described in most of the biosystems [3]. In addition, it does not contribute to the toluene removal and constitute an increase of alternative carbon sources for cryptic growth [26].

### 3D Isosurface and Dynamics of Living and Dead Cells Within the Biofilm

Image processing and analysis methods are widely used in microbial research to quantify and/or semiquantitatively characterize microorganisms growing in biofilms. This was carried out with the specific aim of visualizing the distribution of live and dead cells in the biofilms stained with SYTO9 and PI. This visualization was performed to illustrate the components of these biofilm communities. With regard to biofilm development and fluorescent staining, each of these experiments was performed in triplicate and a representative example of each was visualized using computer graphics (CG), as shown in the data presented here. The 3D isosurfaces of the developing biofilm are shown in Fig. 6.



**Fig. 6.** Distribution of bacterial live cells/dead cells in the developed biofilm (different phases A, B, C, D, and E), presented as a 3D visualization of the biofilm structure as live (green) and dead (red).

In summary, 3D visualization illustrates the dynamics of living and dead cells. The biofilm contained significant proportion of dead cells in phase B. These dynamics are well in agreement with the removal efficiency of the toluene throughout the experiment.

It can be concluded that the above findings lead to a further understanding of the bacterial biofilm dynamics of a membrane reactor operated under different operating

conditions. An optimum toluene removal of 89% was observed at a loading rate of  $14.4 \text{ kg m}^{-3} \text{ d}^{-1}$ . Comparison of two methods (culture dependent and culture independent) shows that CSLM is a good tool to study the structure and dynamics of the biofilms system. 3D visualization of biofilms illustrated the dynamics in living and dead cells, which are well in agreement with the toluene removal efficiency over time. However, further research has to be

done to show the influence of hydrodynamic conditions on the biofilms.

## Acknowledgments

We thank Professor Frans Van Roy and Wies Decekers of the Department for Molecular Biomedical Research, VIB, Ghent, for the CSLM images. The author acknowledges Ghent University for providing a special research grant (BOF) for doctoral research of Bioengineering: Environmental Technology. We also thank Professor Thomas Neu of Helmholtz Centre for Environmental Research - UFZ, Magdeburg, Germany, for useful discussion on this article.

## REFERENCES

- Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**: 143–469.
- APHA. 1980. Standard methods for the examination of water and waste water. 15th Ed. American Public Health Association, Washington, DC.
- Boulos, L., M. Prevost, B. Barbeau, J. Coallier, and R. Desjardins. 1999. LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup>: Application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J. Microbiol. Meth.* **37**: 77–86.
- Characklis, W. 1981. Fouling biofilm development: A process analysis. *Biotechnol. Bioeng.* **23**: 1923–1960.
- Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott. 1995. Microbial biofilms. *Annu. Rev. Microbiol.* **49**: 711–745.
- Duffy, G. and J. J. Sheridan. 1998. Viability staining in a direct count rapid method for the determination of total viable counts on processed meats. *J. Microbiol. Meth.* **31**: 167–174.
- Jones, W., R. G. Mirpuri, S. Villaverde, Z. Lewandowski, and A. Cunningham. 1997. The effect of bacterial injury on toluene degradation and respiration rates in vapor phase bioreactors. *Wat. Sci. Tech.* **36**: 85–92.
- Kirchner, K., S. Wagner, and H. J. Rehm. 1992. Exhaust gas purification using biocatalysts (fixed bacteria monocultures) – the influence of biofilm diffusion rate (O<sub>2</sub>) on the overall reaction rate. *Appl. Microbiol. Biotechnol.* **37**: 277–279.
- Kumar, A., J. Dewulf, and H. Van Langenhove. 2008. Membrane based biological waste gas treatment. *Chem. Eng. J.* **136**: 82–91.
- Kumar, A., J. Dewulf, M. Luvsanjamba, and H. Van Langenhove. 2008. Continuous operation of membrane bioreactor treating toluene vapors by *Burkholderia vietnamiensis* G4. *Chem. Eng. J.* **140**: 193–200.
- Kumar, A., J. Dewulf, A. Verduyssen, and H. Van Langenhove. 2008. Performance of a composite membrane bioreactor treating toluene vapors: Inocula selection, reactor performance and behavior under transient conditions. *Biores. Technol.* (accepted).
- Lawrence, J. R., D. R. Korber, B. D. Hoyle, J. W. Costerton, and D. E. Caldwell. 1993. Optical sectioning of microbial biofilms. *J. Bacteriol.* **173**: 6558–6567.
- Leuko, S., A. Legat, S. Fendrihan, and H. Stan-Lotter. 2004. Evaluation of the LIVE/DEAD BacLight kit for detection of extremophilic Archaea and visualisation of microorganisms in environmental hypersaline samples. *Appl. Environ. Microbiol.* **70**: 6884–6886.
- Lloyd, D. and A. H. Vigour. 1995. Vitality and viability of microorganisms. *FEMS Microbiol. Lett.* **133**: 1–7.
- Love, N. and P. Grady. 1995. Impact of growth in benzoate and *m*-toluate liquid media on culturability of *Pseudomonas putida* on benzoate and toluate plates. *Appl. Environ. Microbiol.* **61**: 3142–3144.
- Mirpuri, R., W. Jones, and J. D. Bryers. 1997. Toluene degradation kinetics for planktonic and biofilm-grown cells of *Pseudomonas putida* 54G. *Biotechnol. Bioeng.* **53**: 535–546.
- Moller, S., D. Korber, G. Wolfaardt, S. Molin, and D. Caldwell. 1997. Impact of nutrient composition on a degradative biofilm community. *Appl. Environ. Microbiol.* **63**: 2432–2438.
- Neu, T. R. and J. R. Lawrence. 1997. Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. *FEMS Microbiol. Ecol.* **24**: 11–25.
- Okkerse, W., S. Ottengraf, R. Diks, B. Osinga-Kuipers, and P. Jacobs. 1999. Long term performance of biotrickling filters removing a mixture of volatile organics from an artificial waste gas: Dichloromethane and methylmethacrylate. *Bioproc. Eng.* **20**: 49–57.
- Schönduvel, P., M. Sara, and A. Friedl. 1996. Influence of physiologically relevant parameters on biomass formation in a trickle-bed bioreactor used for water gas cleaning. *Appl. Microbiol. Biotechnol.* **45**: 286–292.
- Schroeder, E. D. 2002. Trends in application of gas-phase bioreactor. *Rev. Environ. Sci. Bio/Technol.* **1**: 65–74.
- Singh, R., D. Paul, and R. K. Jain. 2006. Biofilms: Implications in bioremediation. *Trends Microbiol.* **14**: 389–397.
- Tolker-Nielsen, T. and S. Molin. 2000. Spatial organization of microbial biofilm communities. *Microb. Ecol.* **40**: 75–84.
- Van Langenhove, H., I. De Bo, P. Jacobs, K. Demeestere, and J. Dewulf. 2004. A membrane bioreactor for the removal of dimethyl sulphide and toluene from waste air. *Wat. Sci. Technol.* **50**: 215–224.
- Villaverde, S. and M. Fernandez. 1997. Non-toluene-associated respiration in a *Pseudomonas putida* 54G biofilm grown on toluene in a flat vapor bioreactor. *Appl. Microbiol. Biotechnol.* **48**: 357–362.
- Villaverde, S., R. G. Mirpuri, Z. Lewandowski, and W. L. Jones. 1997. Physiological and chemical gradients in a *Pseudomonas putida* 54G biofilm degrading toluene in a flat plate vapor phase bioreactor. *Biotechnol. Bioeng.* **56**: 361–371.