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Response of *Pseudomonas putida* F1 cultures to fluctuating toluene loads and operational failures in suspended growth bioreactors

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Abstract The response of *Pseudomonas putida* F1 to process fluctuations and operational failures during toluene biodegradation was evaluated in a chemostat suspended growth bioreactor. The ability of *P. putida* F1 to rapidly increase its specific toluene degradation capacity resulted in no significant variation in process removal efficiency when toluene load was increased from 188 to 341 g m^{-3} h^{-1}. Likewise, bacterial activity rapidly reached steady state performance (in less than 1.5 h after the restoration of steady state operational conditions) following an 8 h process shutdown, or after episodes of toluene or mineral nutrients deprivation. Process performance was however highly sensitive to pH, as pH levels below 4.5 dramatically inhibited bacterial activity, decreasing severely process robustness and inducing a cycle of periodic process collapses and recoveries. This pH mediated deterioration of bacterial activity was confirmed by further respirometric tests, which revealed a 50–60% reduction in the O_2 consumption rate during the degradation of both toluene and 3-methyl catechol when pH decreased from 5.05 to 4.55. Finally, process robustness was quantified according to methods previously described in literature.

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Introduction

The powerful and versatile enzymatic machinery exhibited by microorganisms renders biodegradable almost all toxic volatile organic contaminants (VOCs) present in waste gaseous emissions (Elena and Lenski 2003; Parales and Haddock 2004). Thus, despite its high toxicity, direct or cometabolic mineralization of toxic VOCs has been extensively reported in numerous species of bacteria and fungi (Jung and Park 2005; Morales et al. 2004). Furthermore, the high efficiency of microbial pollutant uptake mechanisms and catabolic enzymes responsible for pollutant breakdown allows microorganisms to mineralize contaminants even at very low concentrations (Roch and Alexander 1997).

Unlike physical/chemical treatment methods, biological off gas treatment methods do not generate secondary pollution (organic pollutants are transformed into innocuous CO_2, H_2O and biomass) and are less energy intensive as they work under mild conditions of pressure and temperature (Kennes and Thalasso 1998; Van Groenestijn and Hesselink 1993). However, despite the above mentioned advantages, biotechniques for air pollution control are not yet fully accepted among process and environmental engineers. Bad practices and limited process control for especially water activity and

pH during the operation of biofilters treating VOCs or H_2S have resulted in poor process performance as a result of bed compaction, excessive biomass overgrowth, or inhibition of the pollutant degrading microbial communities. These episodes have ineluctably contributed to a loss of acceptance among environmental scientists (Maestre et al. 2007; Williams and Miller 1992). In this context, more energy intensive and less environmental friendly treatment methods such as incineration or chemical absorption have been often preferred in industrial applications due to their well established robustness. Process robustness for a biological process can be defined as the ability of the system to deal with fluctuations, and to recover after operational failures (Kraakman 2003, 2005). The quantification of process robustness can improve both the design and operation of the bioreactor by identifying the critical operational variables, which can ultimately lead to a greater acceptance of biological air pollution control reactors. Little information is however available in literature on the robustness of biological gas treatment methods, with most of these studies being focused exclusively on some biofilters (Baquerizo et al. 2007; Boudreau and Daugulis 2006; Maestre et al. 2007; Metris et al. 2001). More research is therefore needed to evaluate the robustness of microbial communities under process fluctuations and operational failures in other bioreactor configurations.

In this work, the ability of *Pseudomonas putida* F1 to deal with fluctuations and to recover from operational failures was investigated during toluene biodegradation in a suspended growth bioreactor (SGR). Chemostat SGRs constitute a valuable tool for evaluating bacterial response to changes in environmental and process parameters. This work was thus devised as a microbiological study rather than a biofiltration process assessment. A *P. putida* strain was selected as model microorganism for being commonly found in air decontamination processes and perhaps the best characterized of the hydrocarbon degrading bacteria (Roy et al. 2003).

Materials and methods

Microorganisms and culture conditions

A *P. putida* F1 strain [DSMZ 6899] was selected for toluene biodegradation purposes. The culture was maintained at 4°C in Mineral Salt Medium (MSM) with toluene as the sole carbon and energy source. To furnish fresh inoculum, 500-ml E-flasks were supplied with 250 ml of MSM, 200 μl of toluene, closed with cotton plugs, sealed with aluminum and parafilm paper, and incubated for 12 h in an orbital shaker at 200 rpm and 30°C.

The MSM used for bacterial cultivation was composed of (g l^{-1}): $NaHPO_4 \cdot 12H_2O$, 6.15; KH_2PO_4, 1.52; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.038; and 10 ml l^{-1} of a trace element solution containing (g l^{-1}): EDTA, 0.5; $FeSO_4 \cdot 7H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.01; $MnCl_2 \cdot 4H_2O$, 0.003; H_3BO_3, 0.03; $CoCl_2 \cdot 6H_2O$, 0.02; $CuCl_2 \cdot 2H_2O$, 0.001; $NiCl_2 \cdot 6H_2O$, 0.002; $NaMoO_4 \cdot 2H_2O$, 0.003. The final pH of medium was 7.0.

Chemicals

All chemicals and reagents were purchased from PANREAC with a purity of +99% (Barcelona, Spain). Analytical grade benzyl alcohol (BA) and 3 methyl catechol (3-MC) were purchased from Sigma-Aldrich (USA). Synthetic air (N_2/O_2, 79/21%) was used during the entire experimentation (Carburos Metálicos S.A., Spain).

Experimental design

The influence of fluctuations and operational failures on process performance was investigated under sterile conditions in a magnetically stirred 1-l glass bioreactor (Afora S.A., Spain) operated as a chemostat (Diaz et al. 2008). In all tests, the bioreactor was filled with 900 ml of sterile MSM and inoculated with 40 ml of *P. putida* F1 to attain initial biomass concentrations ranging from 14 to 38 mg Dry Weight l^{-1} (from now on mg DW l^{-1}). The system was initially operated in batch mode and continuous operation readily established 10 h following inoculation by continuous MSM supply and overflow under sterile conditions using peristaltic pumps (Watson Marlow Bredel, USA). Reactor's liquid volume was however maintained at 900 ml during continuous operation. Temperature and agitation rate were maintained constant at 25°C and 500 rpm, respectively, while no pH control was implemented during the entire experimentation period. Toluene was supplied in the gas phase through the aeration (1,100 ml min^{-1} of synthetic air filtered

through a 0.2 μm Millex®—FG membrane filter) by mixing a toluene-saturated stream with a toluene-free air stream at different proportions. Four series of experiments were carried out under high toluene loadings corresponding to common fluctuations and operational failures during the operation of SGRs. In each series, the process was allowed to reach a stable steady state before inducing the corresponding operational failure or fluctuation. Initial operational conditions were then restored and the transient bacterial response monitored. This experimental protocol was repeated twice within each run in order to determine the reproducibility of process response. In each series, gaseous toluene and CO_2/O_2 concentrations were periodically monitored by simultaneous withdrawing 250 μl gas samples with Gas-Tight Hamilton syringes. Excreted metabolites (i.e. Benzyl Alcohol), dissolved total organic carbon (TOC) concentration, pH, ATP concentration, and absorbance at 650 nm were also periodically recorded by withdrawing a 20 ml liquid sample under sterile conditions. In addition, Dissolved Oxygen Concentration (DOC) and Temperature (T) were monitored on line. In the following, process stability was mainly evaluated based on process removal efficiency (RE), elimination capacity (EC), carbon dioxide production, and specific ATP content as defined in Diaz et al. (2008).

Fluctuations in toluene loadings

Bacterial response to surges on pollutant loading was evaluated. The SGR was initially operated at a dilution rate (D) of 0.2 h^{-1} and a toluene inlet concentration of 2.6 ± 0.2 g m^{-3} (corresponding to a toluene loading of 188 ± 15 g m^{-3} h^{-1}), reaching a steady state after 48 h of operation. At that point, toluene inlet concentration was increased to 4.8 ± 0.3 g m^{-3} (corresponding to a toluene loading of 341 ± 20 g m^{-3} h^{-1}) during 3 h and decreased back to 2.6 ± 0.2 g m^{-3} afterwards. A similar step change was applied 3.5 h after restoring the initial pollutant concentration. Process monitorization was carried out by sampling every 30 min.

Process shutdown

The ability of *P. putida* F1 to recover from an 8 h process shutdown (neither toluene laden air nor MSM were introduced into the process) was investigated. The process was allowed to reach a steady state at D of 0.11 h^{-1} and 6.5 ± 0.4 g m^{-3} of inlet toluene concentration (corresponding to a toluene loading of 455 ± 30 g m^{-3} h^{-1}), and afterwards, both the supply of toluene contaminated air and MSM were suppressed during 8 h. Both toluene loading and MSM dilution rates were then restored to their original values, allowing the system to stabilize during 40 h before the same shutdown protocol was applied again.

Toluene starvation

The ability of *P. putida* F1 to carry out toluene biodegradation after an 8 h starvation period was investigated in order to simulate episodes of low toluene concentrations. Toluene inlet concentration was reduced to zero (worst case scenario) while maintaining both air and MSM supply in a system initially operated at 5.6 ± 0.5 g m^{-3} of inlet toluene concentration and D of 0.1 h^{-1}. After 8 h of bacterial starvation, toluene supply was restored to 5.6 ± 0.5 g m^{-3} and the transient bacterial response periodically recorded.

Stoppage on MSM supply

Bacterial response during episodes of no nutrient supply was evaluated under high toluene loadings (232 ± 45 g m^{-3} h^{-1}). MSM supply was stopped during 8 hours in a SGR working under steady state conditions at D of 0.19 h^{-1} and 3.3 ± 0.7 g toluene m^{-3}. The process was allowed to equilibrate during 100 h before the next restriction in MSM supply.

Respirometric tests

Respirometric assays (15 min assays) were carried out in order to test the influence of pH on O_2 consumption during toluene and 3-MC (the first intermediate of the TOD degradation pathway) biodegradation. Reaction vessels were filled with 15.5 ml of MSM at 3 different pH, 2 ml of *P. putida* F1 (previously centrifuged at 6,000 rpm during 15 min and resuspended in fresh MSM) and supplied with either toluene or 3-MC at 25 mg l^{-1} (from a toluene saturated MSM or a 3-MC stock solution). In

each series of experiments, bacterial cultivation was carried out at pH 6.05, 5.05, and 4.55. The total reaction volume was 18.5 ml. Tests in the absence of toluene or 3-MC at the 3 evaluated pH were also carried out under similar conditions to serve as controls for endogenous respiration.

Process robustness

According to Kraakman (2003), the risk of a system in regards to an individual upset was quantified by determining the negative effect for this upset and multiplying by its frequency. The overall robustness (R) of the process will be then determined as the sum of the risks for each individual upset:

$$R = \sum p \times E \quad (1)$$

p = Probability of occurrence of an upset [=] number of occurrence per year. E = negative effect of the upset [=] % of loss in the total removal (kg/year) provoked by each individual upset.

Analytical procedures

Toluene analysis was performed in a Gas Chromatograph (Hewlett-Packard 5890, Palo Alto, USA) coupled with a Mass Spectrometer Detector (Hewlett-Packard 5973 MSD, Palo Alto, USA) and a HP-5MS fused silica capillary column (Agilent Technologies, USA). Oven Temperature was maintained at 50°C during 3 min and increased up to 90°C at 30°C min^{-1}. Injector and MS Quadrupole were maintained at 250 and 150°C respectively. Helium was used as carrier gas at 0.9 ml min^{-1}. External standards prepared in volumetric bulbs (Sigma-Aldrich, USA) were used for toluene quantification.

CO_2 and O_2 concentrations were measured using a GC-TCD (Agilent Technologies 6890N, Palo Alto, CA, USA) equipped with a PORAPAK N, 80/100 3 m × 1.8 (Tecknokroma, USA) and a Molecular sieve 13 × 45/60 0.9 m × 1/8 (Tecknokroma, USA). Helium was used as a carrier gas at a flow rate of 5.4 ml min^{-1}. The temperatures of the detector and injector were maintained at 200 and 150°C, respectively. The oven temperature was initially maintained at 40°C during 3 min, increased at 40°C min^{-1} up to 100°C, and maintained at 100°C during 1.5 min. External standards enabled CO_2 and O_2 quantification.

Bioreaction metabolites (i.e. BA, Bordel et al. 2007) were quantified by HPLC-UV using a WATERS 515 HPLC pump coupled with a UV1000 Spectraseries Detector (Thermo separation Products, California, USA) and a Supelcosil LC-PAH column (Supelco, Bellefonte, USA). Samples were eluted isocratically using a mobile phase composed of methanol and water (60/40 v/v) at a flow rate of 0.5 ml min^{-1}. UV-detection was performed at 254 nm. Liquid samples of 1.5 ml were centrifuged at 4°C for 10 min at 13,300 rpm prior to analysis. External standards were used to enable quantitative determination.

Dissolved total organic carbon (TOC) in the aqueous phase was measured using a TOC analyzer (Shimadzu TOC-5050A, Japan) according to the manufacturer. Liquid samples of 7 ml were centrifuged at 6,000 rpm during 20 min prior to analysis. Ammonia concentrations in the liquid phase were determined using an Ammonia Electrode, Orion 900/200 (Thermo Electron Corporation, Beverly, USA).

The DOC and the temperature in the bioreactor were determined using an O_2 transmitter 4100 (Meter Toledo GmbH, Urdolf, Germany). A CRISON micropH 2002 (Crison Instruments, Barcelona, Spain) was used for pH determination.

Absorbance at 650 nm was used as an indicator of microbial growth and measured using a HITACHI U200 UV/visible spectrophotometer (Hitachi Ltd, Tokyo, Japan). A correlation between absorbance at 650 nm and *P. putida* F1 dry weight was performed according to Standard Methods (1998). ATP was measured using a Microbial ATP kit HS (Biothema, Stockholm, Sweden) and a Microtox 500 luminometer (Azur Environmental, Carlsbad, Germany).

Respirometric assays were performed in a STRATHKELVIN STRATHOX (Strathkelvin Instruments Limited, Glasgow, UK) according to the manufacturer.

Results

Fluctuations in toluene loadings

When the process was operated at 0.19 h^{-1} and 2.6 ± 0.2 g toluene m^{-3} a steady state was rapidly reached within the first 24 h of cultivation. At that point toluene removal efficiency (RE) and elimination

capacity (EC) remained stable at 83 ± 1% and 154 ± 15 g toluene m^{-3} h^{-1}, respectively. When increasing toluene concentration at the second day of operation (from 49 to 52 h) up to 4.8 ± 0.3 g m^{-3}, both RE and EC increased to stable values of 86 ± 2% and 298 ± 30 g toluene m^{-3} h^{-1}, respectively, within the first 30 min, together with a slight increase in toluene outlet concentration from 0.5 to 0.7 g m^{-3} (Fig. 1). CO_2 production rates also increased from 318 ± 23 to 585 ± 39 g m^{-3} h^{-1} concomitantly with the increase in toluene EC. In this context, DOC rapidly dropped from 48% to 13% within the first hour of operation at high toluene loading. Biomass concentration steadily increased from 590 to 1,027 mg DW l^{-1} during the 3 h loading shock. Likewise, both NH_4^+ concentration and pH gradually decreased along with the increase in biomass concentration. Fluctuations in toluene concentration did not influence significantly neither the TOC concentration nor the specific ATP content, which remained approx. constant at 116 ± 23 mg C l^{-1} and 3.9 ± 0.8 × 10^{-6} mol ATP g DW^{-1}, respectively. Similar results were obtained during the second step change in toluene loading (from 55 to 58 h; Fig. 1).

Process shutdown

A stable steady operation characterized by RE and EC of 84 ± 4% and 372 ± 21 g m^{-3} h^{-1}, respectively, was achieved at $D = 0.11$ h^{-1} and 6.5 ± 0.4 g toluene m^{-3}. The 8-h process shutdown (suppression of toluene, air, and MSM supply at 87 and 135 h following inoculation) caused both a 32% reduction of bacterial ATP content, and a 48% increase in TOC concentration (Fig. 2). Maximum process performance was rapidly restored, reaching steady state RE and EC within the first hour after process start-up (82% and 380 g m^{-3} h^{-1}, respectively). Likewise, both CO_2 production rates and specific ATP concentrations rapidly achieved steady state levels (556 ± 3 g m^{-3} h^{-1} and 3.6 ± 0.1 × 10^{-6} mol ATP g DW^{-1}, respectively). Unexpectedly, TOC concentration increased from 150 to 290 mg l^{-1} during process shutdown. On the other hand, neither pH, NH_4^+, nor biomass concentrations were significantly influenced by process shutdown (Fig. 2). A second process shutdown 2 days later at 135 h resulted in a similar system response.

Toluene starvation

Toluene biodegradation rapidly recovered from a 8 h starvation period, achieving ECs of 337 ± 18 g m^{-3} h^{-1} and CO_2 production rates of 543 ± 15 g m^{-3} h^{-1} within the first hour after the resumption of toluene supply, rates comparable to the steady state values recorded before bacterial starvation (368 ± 19 and 685 ± 68 g m^{-3} h^{-1}) (Fig. 3). DOC increase to 100% saturation in the absence of toluene, and sharply dropped after toluene supply was reestablished. Biomass concentration gradually decreased in the absence of toluene supply from 1461 ± 18 to 561 ± 24 mg DW l^{-1}, and increased thereafter,

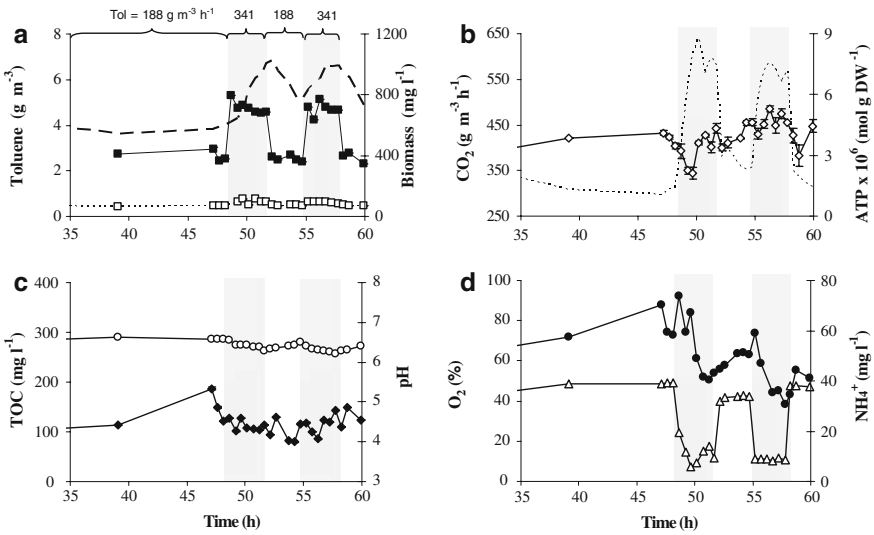

Fig. 1 *P. putida* F1 response to a step change (shaded areas) in pollutant loading from 188 to 341 g m^{-3} h^{-1} during toluene biodegradation in a SGR chemostat. Time course of: (**a**) toluene inlet (■), outlet (□), and biomass concentrations (dashed line); (**b**) specific ATP content (◇) and CO_2 production rate (dotted line); (**c**) TOC (♦) and pH (○); (d) DOC (Δ) and NH_4^+ concentration (●). Numerical values in Fig. 1a represent the toluene mass loading rates applied

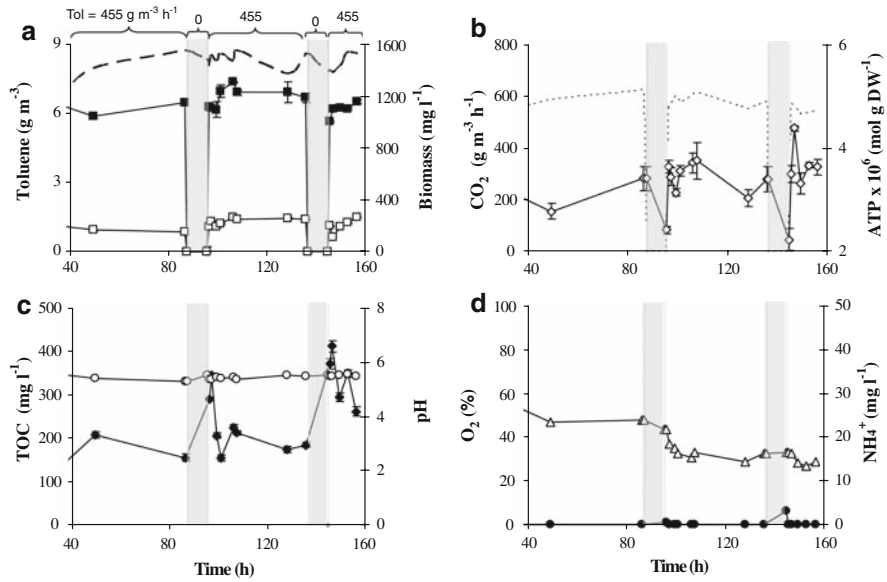

Fig. 2 *P. putida* F1 response to an 8 h process shutdown in a toluene degrading chemostat SGR. Time course of: (**a**) toluene inlet (■), outlet (□), and biomass concentrations (dashed line); (**b**) specific ATP content (◇) and CO_2 production rate (dotted line); (**c**) TOC (♦) and pH (○); (**d**) DOC (Δ) and NH_4^+ concentration (●). Shaded areas represent the 8 h process shutdown and the numerical values in Fig. 2a the toluene mass loading rates applied

reaching steady state concentrations 6 h after toluene supply was resumed. On the other hand, the absence of toluene supply provoked a decrease in both the specific ATP content and TOC concentration from $3.3 \pm 0.1 \times 10^{-6}$ to $7.3 \pm 0.1 \times 10^{-7}$ mol ATP g DW^{-1}, and from 412 ± 4 to 156 ± 2 mg C l^{-1}, respectively. However, while the specific ATP quickly recovered reaching $4.8 \pm 0.2 \times 10^{-6}$ mol ATP g DW^{-1} after 1.5 h under steady toluene supply, TOC remained constant at approximately 200 mg C l^{-1}. In addition, both NH_4^+ and pH gradually increased during process starvation, and declined after the reestablishment in toluene supply down to values of 0 mg N–NH_4^+ l^{-1} (under the detection limit of the NH_4^+ sensor) and 4.3, respectively.

However, despite the rapid recovery of process performance, the system collapsed approximately 7 h after the reestablishment of toluene supply. Process deterioration was characterized by a sudden increase in DOC up to saturation levels, concomitantly with a decline in toluene ECs, CO_2 production rates, biomass concentration and bacterial ATP content (Fig. 3). On the other hand, both pH and NH_4^+ steadily increased during process collapse. The system was capable to recover maximum process performance 20 h after process collapse without further modification in the operational variables. However, this pseudo steady state was soon followed by a similar episode of temporary loss in the toluene biodegradation capacity of *P. putida* F1. Afterwards, process operation underwent a dynamic of periodic culture collapse episodes followed by a rapid recovery of bacterial activity (Fig. 3). Based on the previously reported poor toluene dioxygenase activity under nitrogen limiting conditions in *P. putida* strains (conditions prevailing during process collapse), the concentration of nitrogen in the MSM was increased 3 folds after the 3rd bacterial collapse (day 7) in an attempt to restore a stable process performance (Jenkins and Heald 1996). Steady state ECs were achieved 23 h after the 3rd process collapse but despite the supply of the nitrogen enriched MSM, *P. putida* F1 activity dramatically deteriorated at day 8. At that point both nitrogen and trace element concentrations were increased in the MSM (3 folds) in order to determine whether the deterioration in *P. putida* enzymatic activity was due to medium depletion in any trace element. In this context, bacterial activity increased within 24 h, and declined at day 9 of operation. Finally, process dilution rate (regular MSM) was increased from 0.11 to 0.2 h^{-1}, resulting in stable process operation (Fig. 3).

Stoppage on MSM supply

No significant variations in EC, RE and CO_2 production rate were recorded during the first 3 h of operation in the absence of MSM supply. However, process performance severely declined after 5 h (Fig. 4) and

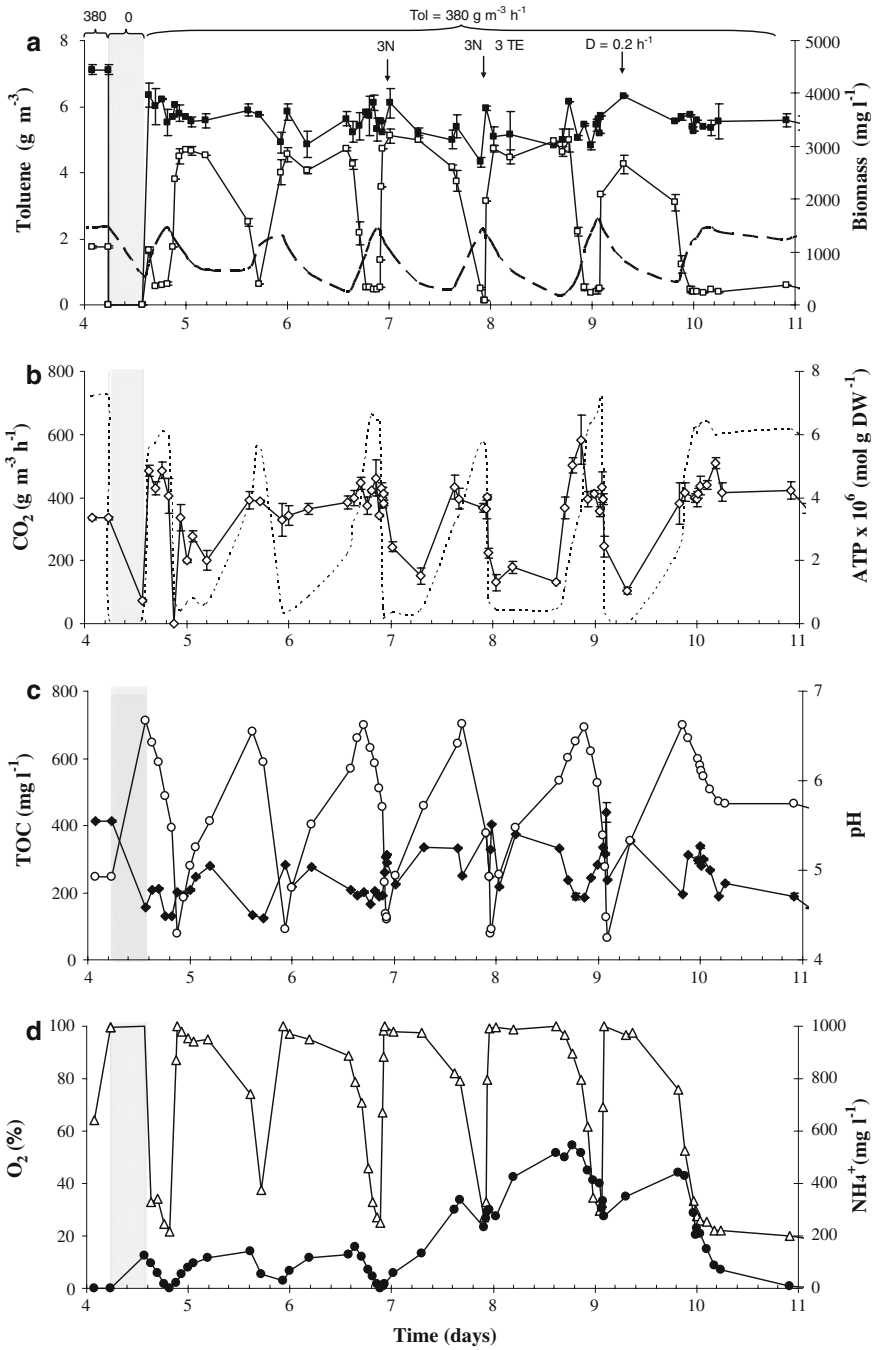

Fig. 3 Time course of bacterial response in a SGR chemostat deprived from toluene during 8 h: (**a**) toluene inlet (■), outlet (□), and biomass concentrations (dashed line); (**b**) specific ATP content (◇) and CO_2 production rate (dotted line); (**c**) TOC (♦) and pH (○); (**d**) DOC (Δ) and NH_4^+ concentration (●). Shaded areas represent operation periods in the absence of toluene and the numerical values in Fig. 3a the toluene mass loading rates applied. Arrows in Fig. 3a represent the introduction of a N enriched MSM (3 N), MSM enriched in N and trace elements (3 N, 3TE), and process operation at dilution rates of 0.2 h^{-1}

continued decreasing despite the reestablishment in MSM supply. Indeed, while steady state ECs ranged from 200 to 220 g m^{-3} h^{-1}, toluene ECs as low as 3 g m^{-3} h^{-1} were recorded 9 h after MSM supply was resumed. DOCs were always negatively correlated to ECs. Thus, during the first hours after process start-up DOC remained constant at approximately 40%, and it suddenly increased up 100% during process collapse (Fig. 4). The specific ATP content of *P. putida* F1 remained constant at $3.5 \pm 0.8 \times 10^{-6}$ mol ATP g DW^{-1} during the first hours of cultivation in the absence of nutrient supply, and decreased concomitantly with the EC down to $1.1 \pm 0.2 \times 10^{-7}$ mol ATP g DW^{-1}. Biomass concentration increased in the

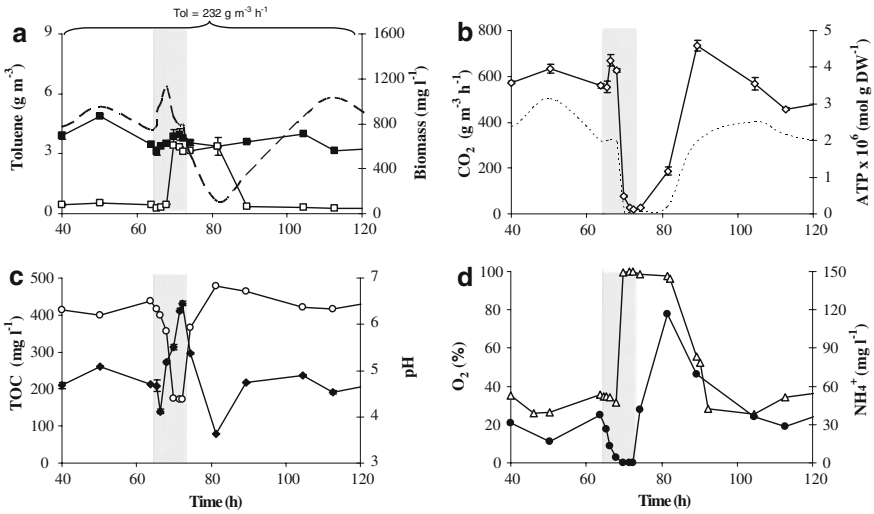

Fig. 4 Time course of toluene biodegradation after a 8 h suppression of MSM supply in a SGR chemostat: (**a**) toluene inlet (■), outlet (□), and biomass concentrations (dashed line); (**b**) specific ATP content (◇) and CO_2 production rate (dotted line); (**c**) TOC (♦) and pH (○); (**d**) DOC (Δ) and NH_4^+ concentration (●). Shaded areas represent operation in the absence of MSM supply and the numerical values in Fig. 4a the toluene mass loading rates applied

absence of dilution, and sharply decreased after MSM addition was restored. Both pH and NH_4^+ concentration steadily decreased from 6.5 to 4.3 and from 36 to 0 mg N–NH_4^+ l^{-1}, respectively, within the first 5 h of cultivation in the absence of MSM supply, and started to increase along with the introduction of fresh MSM (Fig. 4). On the other hand, TOC always correlated toluene EC. Steady state conditions were only recovered 18 h after the reestablishment in MSM supply.

Respirometric tests

Maximum toluene respiration rates of 1.2 gO_2 g DW^{-1} h^{-1} were achieved at pH 5.05 (Table 1). When toluene biodegradation occurred at pH 6.05 and 4.55 specific O_2 consumption rates decreased by 15% and 51%, respectively. Similar results were obtained during the oxidation of 3-MC by *P. putida* F1. Thus, maximum O_2 consumption rates of 0.81 gO_2 g DW^{-1} h^{-1} were recorded at pH 5.05, with reductions of 17% and 60% at pH 6.05 and 4.55, respectively. On the other hand, endogenous respiration rates did increase with increasing pH and remained always one order of magnitude lower than toluene and 3-MC respiration rates.

Table 1 Respiration rates of *P. putida* F1 cultures growing on 25 mg l^{-1} of toluene or 3-MC as the sole carbon and energy source under different pH

pH	Toluene		3-MC	
	Test	Control	Test	Control
4.55	0.595	0.045	0.325	0.053
5.05	1.214	0.070	0.815	0.059
6.05	1.029	0.076	0.678	0.095

Controls were performed in the absence of carbon and energy source. All respiration rates are expressed in (gO_2 g DW^{-1} h^{-1})

Process robustness

Process robustness was quantified according to the method devised by Kraakman (Kraakman 2003). For instance, the risk of the SGR towards daily 8-h process shutdowns can be calculated by determining its probability of occurrence (p, estimated at 365 shutdowns per year) and multiplying by its negative effect. The negative effect (E) caused by each shutdown can be determined by the following equation:

$$\frac{\text{Loss in total removal due to upset}}{\text{Total removal per year}} \times 100 \qquad (2)$$

where the total removal per year can be calculated assuming that bioreactor is operated 16 h a day, 365 days a year treating 0.066 m^3/h (i.e. 385.44 m^3 per year) of a toluene laden air containing 0.0065 kg toluene m^{-3} at 82% RE (Eq. 3):

$$385.44\ \frac{m^3}{\text{year}} \times 6.5 \cdot 10^{-3} \frac{\text{kg toluene}}{m^3} \times 0.82\ \frac{\text{Toluene removed}}{\text{Inlet toluene}} = 2.05\ \frac{\text{kg toluene}}{\text{year}} \qquad (3)$$

Likewise, the loss in total removal due to the 8-h process shutdown must consider the loss during the

first hour after process restart-up (the time experimentally observed to recover steady state conditions), where RE is assumed to increase linearly from 0% to 82%, which translates into a loss of half (41%) of the steady state RE (82%).

$$0.066\frac{\mathrm{m}^3}{\mathrm{h}} \times 6.5 \cdot 10^{-3}\frac{\mathrm{kg\ toluene}}{\mathrm{m}^3} \times \left[0.82 - \frac{(0.82-0)}{2}\right] \times 1\mathrm{h} = 1.8 \times 10^{-4}\ \mathrm{kg\ toluene} \quad (4)$$

According to Eq. 2 a negative effect (E) of 0.009% is obtained. This E multiplied by its probability of occurrence (assumed to be 365 days a year) results in a risk of 3.28%. Table 2 shows the estimated individual risks for the different upsets evaluated in this work and the robustness of the process according to Eq. 1.

Discussion

No significant decrease on toluene RE was recorded when increasing pollutant loading rate from 188 to 341 g m^{-3} h^{-1}, which suggest that the process was limited by pollutant mass transport from the gas to the aqueous phase. Indeed, a higher toluene inlet concentration increased the toluene transfer driving force, with the subsequent enhancement on pollutant uptake rates by *P. putida* F1. The specific toluene removal rates rapidly increased from 0.25 to 0.46 g toluene g DW^{-1} h^{-1} within the first 30 min of operation at high toluene concentrations, which reveals the high catabolic potential of this microorganism. This increase in *P. putida* activity did not cause however a higher energetic level (represented by the specific ATP content) as shown by the constant ATP content recorded before and after the step change in toluene concentration. This observation is in agreement with the results of Bordel et al. (2007) who correlated the specific ATP content in *P. putida* F1 with the aqueous toluene concentration supporting bacterial growth, which in this experiment remained almost constant despite the fluctuations in pollutant loading (2.2 vs. 3.1 mg l^{-1}). In addition, the fact that the recorded two folds increase in EC brought about a two folds increase in CO_2 production, an increase in O_2 consumption, and a constant TOC concentration, confirmed that toluene was mineralized rather than biotransformed into bioreaction intermediates (Fig. 1). Previous studies have also revised the influence of sudden fluctuations of pollutant loadings on the performance of biofiltration systems treating both VOCs and H_2S (Barona et al. 2004; Kraakman 2003, 2005). In most cases, biofilters exhibited a highly effective response in a relatively short period of time (Halecky et al. 2007). Under comparable experimental conditions (60 s empty bed residence time, and 22°C), Metris et al. (2001) observed a decrease of 20% in toluene RE in a compost-perlite biofilter when the concentration of a toluene-xylene laden effluent fed to the process was increased from 0.6 to 2.5 g C m^{-3}. Likewise, Halecky et al. (2007) recorded a reproducible decrease in RE from 98% to

Table 2 Risk estimation for the different upsets considered in this work

Upsets (Deviations from steady state operation)	Example	Probability (P) No. per year	Negative effect (E) Loss in toluene removed (%)	Risk ($p \times E$) (%)
Fluctuations in toluene concentration	Increase of the average toluene concentration by 100%	All the time	0	0
Process shutdown	Production stop (8 h)	365	0.009	3.28
No MSM supply/no pH control	Broken valve/temporary no water source	1	0.18	0.18
No MSM supply/pH control	Broken valve/temporary no water source	1	0	0
No toluene/no pH control	Production stop with air supply to the bioreactor (8 h)	365	0.135	49.27
No toluene/pH control	Production stop with air supply to the bioreactor (8 h)	365	0	0
Total risk	No pH control			52.73
	pH control			3.28

62% when styrene concentration was increased for 40 min from 50 to 200 mg m^{-3} in a biofilter packed with perlite and inoculated with an enriched mixed bacterial culture.

An 8 h process shutdown did not cause a significant deterioration in the short term toluene biodegradation performance. Both the energetic cellular level (reduced as a result of starvation), and the O_2 consumption or the toluene mineralization rapidly attained steady state values in less than 60 min (Fig. 2). TOC concentration increased during process shutdown (Fig. 2c), due to the excretion of extracellular organic matter (metabolites, cell lysis products) since no external carbon source was amended to the bioreactor during that period, although the reason for this excretion remains unclear. The impact of a temporary process shutdown on the performance of four conventional biofilters treating toluene was also investigated by Maestre et al. (2007). The authors found that when biofilters were packed with organic materials, such as compost or pine leaves, toluene biodegradation was not significantly affected by a 5 days shutdown. These results confirmed the observations of Moe and Qi (2004) who reported a recovery period of 5 days in a conventional biofilters packed with polyurethane foam after a 2 days process shutdown.

The reestablishment of toluene supply after an 8 h starvation period under continuous MSM addition quickly restored both O_2 consumption, CO_2 production, and the specific ATP content (Fig. 3). Despite the low biomass concentration present after toluene starvation (as a result of biomass dilution), toluene EC rapidly reached steady state levels due to the ability of *P. putida* F1 to increase its toluene specific consumption rate (from 0.26 to 0.43 g toluene g DW^{-1} h^{-1}). It is therefore remarkable that even during the treatment of high toluene loadings (466 ± 50 g m^{-3} h^{-1}) *P. putida* F1 did not employ its full biodegradation potential, which confirms the great potential of this strain in bioremediation applications. However, despite the short term response of the system, process collapse occurred and recovered 20 h after. Bioreactor operation entered then in a phase of periodic bacterial collapses and recoveries. In order to elucidate the source of the deterioration in bacterial activity a NH_4^+ enriched MSM was supplied after the 3rd process collapse. The pernicious effect of nitrogen deprivation on *Pseudomonas* during toluene biodegradation has been extensively reported in literature (Nielsen et al. 2006; Jenkins and Heald 1996). However, in our case *P. putida* F1 experienced again a 20 h deactivation despite NH_4^+ was present in the cultivation medium at concentrations higher than 300 mg $N\text{–}NH_4^+$ l^{-1}. An additional supplementation in both trace elements and NH_4^+ also failed to stabilize toluene biodegradation, which ruled out the possibility of a process collapse mediated by a limitation in any of the trace elements. Only the increase in the dilution rates at day 10 (from 0.11 to 0.2 h^{-1}) successfully stabilized toluene biodegradation (Fig. 4). The use of a higher dilution rate increased both the buffer capacity in the system (which stabilized culture pH) and boosted the washout of any potential inhibitory metabolite accumulated during the period of active toluene biodegradation. Additional experiments (not described in M&M section) using the same starvation protocol were then carried out at $D = 0.2$ h^{-1}. Process performance rapidly recovered and stabilized after the resumption in toluene supply (Fig. 5). In addition, the same protocol was repeated using a dilution rate of 0.1 h^{-1} and a highly buffered MSM (pH 7.31). Process performance also recovered after bacterial starvation in very short period of time and without further process collapse. In both experiments, process response was reproducible when repeating the test after process stabilization under the same operational conditions (Fig. 5). These results therefore confirmed that pH, rather the accumulation of any toxic metabolite, was the main factor inducing process collapse after an 8 h starvation period. This deterioration of bacterial activity was further confirmed by the respirometric tests performed, which revealed a 50–60% reduction in the O_2 consumption rate during the degradation of both toluene and 3-MC at pH 4.55 when compared to those recorded at pH 5.05. The lower respiration rate of 3-MC when compared to toluene respiration at comparable concentrations suggests the potential accumulation of 3-MC during the long term run. However, accumulation of 3-MC in *P. putida* F1 cultures was recorded neither in this study nor in previous experiments carried out in our lab (Diaz et al. 2008). In addition, it must be highlighted that both the occurrence of severe pH shifts and pH mediated effects was totally unexpected based on the high stability shown by *P. putida* F1 in a similar experimental set-up during

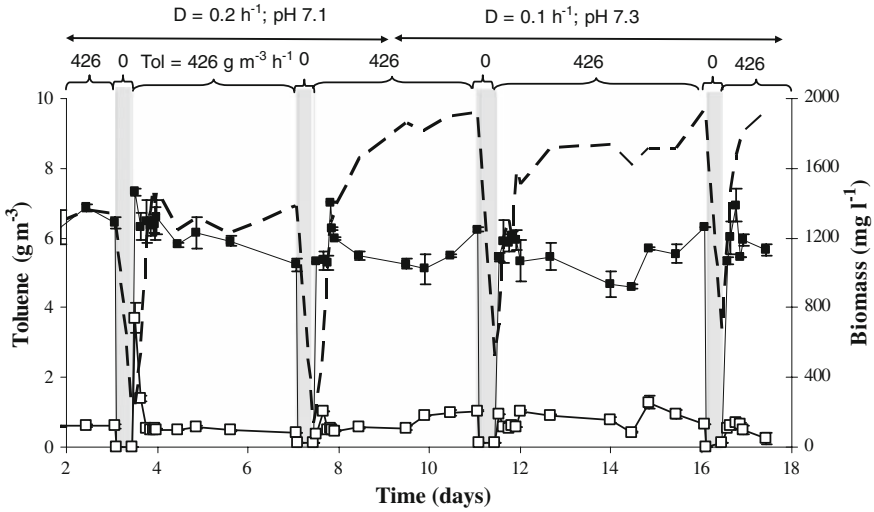

Fig. 5 Time course of toluene inlet (■), outlet (□), and biomass concentrations (dashed line) in a chemostat SGR deprived from toluene during 8 h. The system was operated at $D = 0.2\ h^{-1}$ and inlet pH 7.1 during the first 8 days and at $D = 0.1\ h^{-1}$ and inlet pH 7.31 during the rest of the experiment. Shaded areas represent operation in the absence of toluene and the numerical values the toluene mass loading rates applied

toluene biodegradation at concentrations up 20 g m^{-3} (Diaz et al. 2008).

In the absence of MSM supply toluene biodegradation was not significantly affected during the first 3 h of operation. All parameters monitored except biomass and NH_4^+ concentration, and pH remained within their steady states levels. Biomass concentrations increased due to a sustained toluene mineralization and to the absence of medium dilution. Likewise, both pH and NH_4^+ gradually decreased down to 4.3 and 0 mg l^{-1} during the 5 h following the suppression of MSM supply. At that point, process collapse likely due to a pH mediated inhibition on *P. putida* activity occurred. As expected taking into consideration the high buffer capacity of the system at D of 0.19 h^{-1}, process recovered 18 h after the resumption of MSM and stabilized. These results highlight one more time the need to implement a pH control strategy during the treatment of high toluene loading rates.

In summary, the rapid bacterial response together with the high resistance and powerful enzymatic machinery of *P. putida* F1 resulted in a relatively fast recovery of steady state conditions under the fluctuations and operational failures evaluated. Special attention should be given to pH control as suboptimal pH levels dramatically decreased bacterial activity, decreasing notoriously process robustness. The analysis of process robustness based on Kraakman methodology (Table 2) showed that the overall risk of the bioreactor could decrease from 52.73% to 3.28% by simply installing a pH control unit, thus turning the process into a reliable method for the abatement of toluene. Further work should focus on imposing other and hasher operational fluctuations and failures like fluctuations in temperature, water activity and chances in compound in order to determine its risk to improve process performance.

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