Appl Microbiol Biotechnol (2011) 90:1589–1608
DOI 10.1007/s00253-011-3229-z

MINI-REVIEW

# Overcoming substrate inhibition during biological treatment of monoaromatics: recent advances in bioprocess design

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Received: 28 January 2011 / Revised: 9 March 2011 / Accepted: 9 March 2011 / Published online: 8 April 2011


**Abstract** The biological removal of monoaromatic compounds from contaminated environments, usually arising from industrial activity, is challenging because of the inherent toxicity of these compounds to microorganisms, particularly at the concentrations that can be encountered in industrial waste streams. A wide range of bioprocess designs have been proposed and tested with the aim of achieving high removal efficiencies, with varying degrees of technical success, and potential for practical implementation. This review reports on the progress on variations of well-known themes made in the last 3–4 years, as well as new bioprocess technologies that address the cytotoxicity of monoaromatics directly. Areas for further research are also proposed.

**Keywords** Monoaromatics · Bioprocess designs · Cytotoxicity

## Introduction

Reducing or eliminating inhibition of microbial activity is a critical consideration when designing bioprocesses for the treatment of xenobiotic compounds such as monoaromatics. Substrate inhibition can result in reduced reaction rates, and even complete cessation of microbial activity, as well as amplified toxicity arising from substrate interaction effects in the degradation of xenobiotic mixtures. The inhibition characteristics of environmental pollutants can be expressed in terms of effective concentrations from toxicity analysis (e.g., $EC_{50}$) and inhibition constants derived from dedicated kinetic models such as, for instance, the Haldane equation, which is one of the most commonly used expressions for characterizing substrate inhibition:

$$r_S = k\frac{S}{S + K_s + \frac{S^2}{K_I}}$$

where:

| | |
|---|---|
| $k$ | Kinetic parameter [$ML^{-3}T^{-1}$] |
| $S$ | Substrate concentration [$ML^{-3}$] |
| $K_s$ | Saturation constant [$ML^{-3}$] |
| $K_I$ | Inhibition constant [$ML^{-3}$] |

The partition coefficient of chemicals in an octanol-water biphasic mixture ($K_{ow}$) is also often used to predict the biosorption and acute toxicity potential of chemicals because it is a good indicator of a molecule's solubility in cell membranes. For this reason, the acute toxicity of structurally related chemicals (e.g., chlorophenols) is often positively correlated to their $K_{ow}$ coefficients. Relevant toxicity properties and $K_{ow}$ values for individual BTEX compounds and typical representatives of substituted phenols are presented in Table 1a and b. In practice, wastewaters from olive mills and kraft pulp mills can contain up to 190–350 mg/L chlorophenols 12 g polyphenols/L, and 4.3 g/L phenol (Smets and Barkay 2005; Khoufi et al. 2006), which are far above the toxicity threshold of these contaminants, as shown in Table 1b.

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**Table 1** Toxicity data for BTEX and typical representatives of substituted phenols

| Compound | Water solubility | Log$K_{ow}$ | $EC_{50}$ | | Reference | $K_I$ | | Reference |
|---|---|---|---|---|---|---|---|---|
| | (mg/L) | | Test | (mg/L) | | Biomass | (mg/L) | |
| (a) BTEX | | | | | | | | |
| Benzene | 1785.5 | 2.13 | (1) | 75.00 | Boyd et al. 1997 | *Pseudomanas fluorescens* | 80.0 | Kim et al. 2005 |
| | | | | | | *Pseudomonas putida* | 100.0 | Kim et al. 2005 |
| | | | | | | *Pseudomonas aeruginosa* | 130.0 | Kim et al. 2005 |
| | | | | | | Mixed culture | 142.1–211.8 | Maliyekkal et al. 2004 |
| Toluene | 532.6 | 2.73 | (2) | 91.20 | Parvez et al. 2008 | Mixed culture | 209.4–269.5 | Maliyekkal et al. 2004 |
| Ethylbenzene | 161.5 | 3.15 | (2) | 6.02 | Parvez et al. 2008 | *Pseudomonas* sp. | 161.2 | Veiga et al. 1999 |
| | | | | | | *Bacillus* like sp. | 117.5 | Veiga et al. 1999 |
| *o*-Xylene | 161.5 | 3.20 | (2) | 14.12 | Parvez et al. 2008 | Mixed culture | 134.3–175.6 | Maliyekkal et al. 2004 |
| | | | (2) | 8.83 | Zhao et al. 1995 | | | |
| *m*-Xylene | 171.5 | 3.12 | (2) | 19.31 | Zhao et al. 1995 | | | |
| *p*-Xylene | 181.5 | 3.15 | (2) | 17.22 | Zhao et al. 1995 | | | |
| (b) Phenols and substituted phenols | | | | | | | | |
| Phenol | $83\times10^3$ | 1.46 | (3) | 280.00 | Walker 1989 | *Candida tropicalis* | 185.0 | Adav et al. 2007 |
| | | | (2) | 41.68 | Parvez et al. 2008 | *P. putida* | 172.0–221.0 | Onysko et al. 2000 |
| | | | (1) | 19.00 | | Activated sludge | 72.4 | Marrot et al. 2008 |
| | | | | | | *Alcaligenes* | 152.0–550.0 | Essam et al. 2010 |
| 4-Nitrophenol | $160\times10^3$ | 1.91 | (1) | 13.70 | Somasundaram et al. 1990 | Mixed culture | 34.2 | Tomei and Annesini 2008 |
| | | | (3) | 59.00 | Walker 1989 | | | |
| | | | (4) | 57.00 | Walker 1989 | | | |
| 3,4 Dimethylphenol | $7.2\times10^3$ | 2.77 | (1) | 0.39 | | Mixed culture | 549.8 | Acuña-Argüelles et al. 2003 |
| 2- Chlorophenol | $20\times10^3$ | 2.15 | (1) | 33.81 | | Mixed culture | 1.1–1.7 | Gaudy et al. 1988 |
| | | | (4) | 141.00–163.00 | | | | |
| 4-Chlorophenol | $27\times10^3$ | 2.39 | (1) | 8.30 | Ribo and Kaiser 1983 | Mixed culture | 79.7 | Sahinkaya and Dilek 2007 |
| | | | | | | *Candida tropicalis* | 4.3 | Jiang et al. 2007 |
| 2,4-Dichlorophenol | $4.5\times10^3$ | 3.06 | (1) | 5.04 | Ribo and Kaiser 1983 | Mixed culture | 44.5 | Sahinkaya and Dilek 2007 |
| | | | (4) | 40.00–91.00 | Erol Nalbur and Alkan 2007 | | | |
| 2,4,6-Trichlorophenol | $0.8\times10^3$ | 3.70 | (1) | 7.70 | Ribo and Kaiser 1983 | Mixed culture | 610.0 | Kharoune et al. 2002 |
| *o*-Cresol | $26.8\times10^3$ | 1.99 | (1) | 27 | Santos et al. 2006 | Mixed culture-aerobic granules | 824.0 | Lee et al. 2011 |
| | | | | | | *Arthrobacter* | 800.0 | Kar et al. 1997 |
| *m*-Cresol | $19.6\times10^3$ | 1.98 | (1) | 11 | Chang et al. 1981 | Mixed culture-aerobic granules | 952.0 | Lee et al. 2011 |
| *p*-Cresol | $22.0\times10^3$ | 1.96 | (1) | 1.00 | | Mixed culture-aerobic granules | 617.0 | Lee et al. 2011 |
| | | | | | | *Arthrobacter* | 1050.0 | Kar et al. 1997 |

(1) Microtox, (2) *V. fischeri*, (3) Activated sludge respiration inhibition, (4) OECD

With regard to the inhibitory effect of substrates in mixtures, limited information is available in the scientific literature because the majority of kinetic studies have been performed using single target compounds. A variety of interactive effects are possible with mixed substrates, however, as recently demonstrated by Littlejohns and Daugulis (2008) who identified and quantified the substrate interaction mechanisms of inhibition, enhancement, and cometabolism during the biodegradation of BTEX mixtures by a bacterial consortium.

Every bioremediation process will benefit from the presence of microorganisms with the capability to tolerate and rapidly mineralize the target xenobiotic molecules, and a number of past efforts have studied: microbial tolerance to solvents and monoaromatic compounds (Inoue and Horikoshi 1989; Isken and de Bont 1998; Heipieper et al. 2007), mechanisms for such tolerance (Ramos et al. 2002; Sikkema et al. 1994; Sikkema et al. 1995; Weber and de Bont 1996; Neumann et al. 2005) microbial growth within organic phases (MacLeod and Daugulis 2005) and horizontal gene transfer to provide microbial adaptation to xenobiotics (Smets and Barkay 2005; Top and Springael 2003; Springael and Top 2004). Although improving the microbial properties (e.g., toxicity resistance through gene transfer, selection and/or adaptation) has tremendous potential, the application of "super-bugs" remains limited by stability and versatility issues. For example, long times may be required for stable phenotypes to emerge, and the complexity and variability (in number of components and their concentrations) of the waste streams to be treated can complicate an adaptation process that must respond rapidly to generate an effective and dynamic microbial community. Certainly microbial adaptation plays an important role in the biodegradation of xenobiotic compounds, however, appropriate technological solutions are essential to obtain process performance suitable for real applications; in other words acceptable performance can arise from a combination of the two elements of adaptation and efficient technological solutions. This review focuses on the technology platforms needed to support microbial activity regardless of the intrinsic properties of the microorganisms.

Ex situ bioremediation technologies for the removal of monoaromatics such as BTEX and substituted phenols have been extensively investigated in recent years (Farhadian et al. 2008) and these processes have generally yielded satisfactory removal capability at the laboratory scale. Nevertheless, the widespread application of bioremediation technologies is strongly hampered by the serious inhibitory effects exerted by high substrate concentrations. A particularly challenging application is the treatment of industrial wastewaters, which can be characterized by concentration levels significantly higher (orders of magnitude) than those considered to be inhibitory, as illustrated above. In those cases, modified treatment configurations designed to minimize the impact of toxicity on microbial activity and maximize the volumetric removal rate have been investigated. A variety of approaches have been suggested for this purpose. In the present review, monoaromatic treatment technologies have been grouped into three types: conventional methods, two-phase systems, and integrated chemical–biological treatment, as summarized in Table 2. Reported data refer to research papers published in the last 3–4 years.

In the case of conventional methods, biofilm reactors have been distinguished from immobilized cells reactors by considering that in the former case the biocatalyst grows *onto* a support (attachment mechanism) where it is not completely immobilized (the thickness of the biofilm layer depends on the hydrodynamics of the system) while in the latter case the biomass is confined (i.e., completely immobilized) *into* a support via an entrapment mechanism. A common strategy for dealing with substrate toxicity is to increase the biomass concentration (by, for example, using biofilm, immobilized cell and granular sludge reactors) with the concomitant beneficial effect of increasing the process kinetics and reducing the substrate/biomass ratio. In the case of sequencing batch reactors (SBRs) substrate toxicity is reduced by taking advantage of the "induction" effect of developing alternative metabolic pathways arising from the dynamic operating conditions characteristic of SBR systems. Two-phase systems, now almost universally called "Two Phase Partitioning Bioreactors" (TPPBs), deal directly with substrate inhibition by reducing aqueous phase concentrations via sequestration into a second, immiscible phase, and re-release of the substrate based on metabolic demand and the maintenance of thermodynamic equilibrium. Process alternatives for TPPBs include liquid–liquid configurations, encapsulated liquid systems, and the use of solid sequestering phases, such as polymers. Combined treatment processes utilize synergistic physical/chemical methods to initially modify (detoxify) the monoaromatic substrates, followed by biological treatment, with the aim of enhancing overall performance efficiency.

## Conventional methods

### General strategies for the biological removal of inhibitory substrates

This review focuses on recent findings; well-known biotechnologies (e.g., activated sludge process) commonly used for the treatment of certain toxic effluents will not be considered. However, before presenting the specific features of selected technologies, it is first necessary to briefly summarize the general strategies for the biological treatment of toxic effluents.

**Table 2** Methods and reactor configurations to reduce the impact of toxic substrates

| Method | Reactor configuration | Strategy |
|---|---|---|
| Conventional | Biofilm | Increase of biomass concentration |
| | *Fixed bed* | *High biomass concentration* |
| | *Expanded bed, Fluidized bed, Pulsed bed* | *High biomass concentration, high contact surface* |
| | *Adsorptive support (GAC)* | *Sorption as additional removal mechanism* |
| | Immobilized cell | Confinement, protection and no losses of the biomass |
| | *Membrane, fixed bed* | *High biomass concentration* |
| | *Fluidized bed* | *High biomass concentration, high contact surface* |
| | *Adsorptive immobilizing agent (added of GAC, GAC)* | *Sorption as additional removal mechanism* |
| | Sequencing batch reactors | Dynamic operating conditions, flexibility in operation |
| | Granular sludge | High biomass "density" |
| Two-phase systems | Two-phase partitioning bioreactors | Partitioning of the substrate into sequestering phase, which releases substrate to cells based on their metabolic demand |
| | *Liquid–liquid* | *Organic solvents as partitioning phase* |
| | *Encapsulated* | *Organic solvents in polymer matrices* |
| | *Solid–liquid* | *Polymers as partitioning phase* |
| Integrated chemical/biological treatment | Different reactor configurations depending on the process sequence | Combination of methods: physical/chemical followed by biological treatment |

Microbial kinetic theories suggest that, when effective microorganisms are available, well-mixed biological processes can be operated continuously (or semi-continuously in the case of SBRs) at a dilution rate low enough to keep the concentrations of inhibitory pollutants in the reactor below their inhibition thresholds (or at least below an acceptable limit). A low dilution rate can however be costly, requiring a large reactor volume, or can be difficult to achieve in practice because the low substrate concentration provided to the microorganisms reduces microbial activity and favors the generation of a form of biomass (e.g., filamentous bacteria) that can be difficult to remove and recycle using conventional clarification. Membranes, biofilms, or immobilized biomass can be used to keep a high biomass concentration in the reactor and circumvent this problem. Efficient biomass retention thus allows high volumetric removal rates (i.e., small reactor volumes), even under partially inhibitory substrate conditions in which the high number of microorganisms compensates for low individual activity.

A second issue associated with "conventional" biological treatment is the management of toxic shocks (sudden toxicity pulses) generated by inadvertent changes in wastewater flow or composition. The effects of shocks can be partially and momentarily mitigated using a combination of (1) a buffering or surge tank or controlled substrate delivery (SBR); (2) high mixing to ensure quick substrate dispersion into the reactor volume; (3) diffusion-limited protection using microbial biofilms or granules and/or dedicated carriers, with the efficiency of these strategies depending on the shock intensity and its duration. Because substrate dilution and/or dispersion are needed for the biological treatment of inhibitory effluents, reactors configurations allowing well-mixed conditions should always be preferred regardless the type of biomass retention used (biofilm, attached, recycled). Packed-bed bioreactors, which are traditionally operated as plug-flow, are therefore generally not recommended unless the liquid broth is circulated to provide mixing.

## Biofilm reactors

Biofilm reactors are extensively used in the treatment of industrial wastewater, leachate, and groundwater, and can be operated under aerobic, anoxic, and anaerobic conditions. The growth of biomass onto support materials facilitates high biomass concentrations advantageous for the degradation of poorly biodegradable and/or inhibitory contaminants. Packing media can be inert (plastic, stone, sand, wood, ceramics, etc.) and adsorptive such as the ubiquitous granular activated carbon (GAC). An adsorptive matrix can reduce bulk concentrations of substrates and potentially protect microorganisms by reducing microbial inhibition caused by toxic contaminants, thereby increasing the removal efficiency and also improving the system response to variations in influent contaminant concentration. The use of adsorptive media can however be limited

by the associated costs and by the deterioration of the adsorption capacity caused by the progressive accumulation of the contaminants onto the solid matrix.

Biofilm reactors have been configured as fixed-bed (e.g., biofilters for the treatment of gaseous streams), expanded-bed, or fluidized-bed contactors. The latter two configurations are usually preferred for the treatment of toxic aqueous streams because, as discussed above, they provide well-mixed conditions and higher mass transfer rates that are required at high substrate loadings.

*Fixed bed*

Recent applications of fixed-bed reactors for the treatment of monoaromatics in water have been limited to the removal of phenol (Tziotzios et al. 2007, Bajaj et al. 2009), a compound significantly less toxic than substituted phenols and BTEX (see Tables 1a and b). The operating mode and the specific surface area of the support material can significantly affect reactor performance. For instance, Tziotzios et al. (2007) reported shorter reaction times in a pilot-scale aerobic reactor packed with gravel support media instead of plastic tubes, and a marked positive effect of recirculation on process kinetics. High phenol removal efficiencies (up to 94%) were also achieved in anaerobic fixed bed reactors but these systems were sensitive to influent loads and required 1 month without feed to recover (Bajaj et al. 2009).

Biofilm bioreactors using GAC as the support material were also applied in batch and column studies for the removal of phenol, chlorophenol, and *o*-cresol and modelled assuming fixed bed operation (Quintelas et al. 2010). These authors observed removal efficiencies that were strongly affected by the initial concentrations of the inhibitory substrates. Thus, removal efficiencies were reduced in proportion to the toxicity levels, from 99.5% to 93.4% for phenol, from 99.3% to 61.6% for chlorophenol and from 98.7% to 73.7% for *o*-cresol when the pollutants initial concentrations were increased from 100 to 1,000, 1,600, and 1,700 mg/L, respectively.

GAC was utilized as a support for the attachment of the yeast *Candida tropicalis* in a fluidized bed bioreactor operated in a non-turbulent flow regime to reduce biomass detachment and wash out (Galíndez-Mayer et al. 2008). The reactor was applied to the removal of phenol and 4-chlorophenol at increasing volumetric loadings. The removal efficiency of phenol was strongly affected by the influent load and a drastic decrease was observed for values ≥60 mg phenol/(Lh) while 4-chlorophenol in a mixture with phenol was efficiently degraded at significantly lower volumetric loads in the range of 1–4 mg/(Lh).

As seen above, the operation of fixed-bed reactors can be limited by mass transfer. Therefore, high liquid circulation and oxygen supply rates are needed to continuously and homogeneously supply substrates and to keep the immobilized biomass active and uniformly distributed within the reactor bed. To overcome oxygen mass transfer limitations in submerged fixed-bed systems, Gómez-De Jesús et al. (2009) proposed a prototype of a packed bed bioreactor equipped with a net draft tube riser for liquid oxygenation and recirculation. This bioreactor operates with axial and radial flow by oxygenating the liquid in a wire-mesh draft tube located in the center of the packed bed. The prototype, packed with a porous support of a volcanic stone fragments, was successfully tested for oxygen mass transfer efficiency and was applied to the removal of 2,4,6-trichlorophenol using phenol as the primary substrate. Complete removal of phenol (at an influent concentration 92 mg/L) and 2,4,6-trichlorophenol removal efficiencies ≥98% (at influent concentrations in the range of 25–139 mg/L) were achieved.

*Fluidized bed*

Fluidized bed biological reactors support higher contaminant-biomass and gas–liquid mass transfer than fixed-bed bioreactors. In the case of aerobic processes, this minimizes the formation of anaerobic zones in the deep layers of the biofilm. Moreover, fluidization produces low particle attrition and reduces the hydraulic short-circuiting arising from the formation of preferential flow paths and bed clogging by the growing biomass. Considerable research has been done on the development and testing on of GAC-based fluidized bed bioreactors. Recent investigations on monoaromatics have focused on optimizing operating conditions (Carbajo et al. 2010) and testing innovative biomass carriers (Sevillano et al. 2008). Carbajo et al. (2010) thus reported efficient phenol removal (≥95%) in an anaerobic fluidized bed reactor operated in continuous and batch regimes. However, phenol removal efficiency declined with increasing loading under continuous treatment and with increasing initial concentrations during batch operation. Finally, increased bed viscosity and biomass adhesion to the reactor walls was observed at the highest influent load (3.0 kg/m$^3$ d) tested.

A critical aspect limiting the applicability of fluidized bed reactors is the high energy requirement for fluidization. Therefore, several researchers have investigated the use of alternative attachment media characterized by high specific-surface area and low density. Sevillano et al. (2008) thus proposed a cyclodextrin polymer (β-cyclodextrin cross-linked with epichlorohydrin) as a biofilm carrier. In addition to having a low density, this material possessed favorable sorption properties for phenolic compounds. It is worth noting that this feature can be advantageously exploited only during the start-up phase (or perhaps during transient loadings) because of the saturation of the

cyclodextrin. The system provided good removal efficiency (~90%) at phenol loadings of 0.7–1 kg/m$^3$ d. However, a sudden decrease in removal efficiency was observed when the influent load was increased and was attributed to the inhibitory nature of phenol in combination with the low HRT applied.

Fluidization is often provided by pneumatic agitation during aerobic treatment in order to take advantage of the energy needed for aeration. An alternative technological solution is provided by airlift bioreactors, which are compartmented pneumatically agitated bioreactors containing a riser section, where a gas phase is injected (often air), and a downcomer section containing no or little gas phase relative to the riser. By introducing gas specifically in the riser, a density gradient is established between the liquid phases contained in the riser and downcomer sections, which drives the liquid flow circulation between these sections. This flow regime minimizes shear stress and, consequently, favors microbial growth. Additional advantages of airlift reactors are their simplicity in construction and operation and reduced energy demand. Internal loop airlift reactors were for instance successfully applied to the degradation of phenol by *C. tropicalis* (Feng et al. 2007), and phenol and *m*-cresol in single and dual substrate systems by a mixed microbial culture dominated by *Pseudomonas* sp. (Saravanan et al 2008, 2009). The slower degradation rate observed for *m*-cresol in single-compound tests was attributed to the higher toxicity of this compound. This hypothesis was confirmed by the delayed degradation of *m*-cresol in the mixture tests performed at a high concentration (300 mg/L) of the two compounds.

Airlift bioreactor can be used with free-swimming, flocculated, granulated, attached, or immobilized biomass. They can be used with either fixed (the carriers are generally located inside the downcomer) or fluidized biofilm (the carriers are located inside the riser). In addition, although airlift reactors are generally utilized for aerobic processes, these systems are suitable for integrated anaerobic/aerobic processes by manipulating oxygen transfer in the various reactor sections. For instance, Zhao et al. (2009) applied an internal loop airlift bioreactor for the treatment of phenolic wastewater by adding porous polyurethane microbial carriers. An aerobic zone was thus created in the liquid bulk containing suspended biomass by aeration, whereas an anaerobic zone was formed inside the biofilm. This configuration has been found to improve the flexibility and the spectrum of applications of airlift bioreactors, including the removal of aromatic pollutants. Accurate control of the air flow rate is required, however, to maintain fluidization and high dissolved oxygen levels in the aerobic zone without causing a loss/reduction of anaerobic conditions inside the biofilm.

*Pulsed-plate bioreactors*

An intermediate configuration between the fixed-and fluidized-bed reactors is the recently proposed pulsed-plate bioreactor (Shetty et al. 2007a, b, 2011). Its principle of operation involves the application of a pulsation to a fixed-bed biofilm reactor to improve mass transfer and reduce the problems of preferential fluid paths, short-circuiting and clogging. The slow movements of the bed cause the renewal of the interfacial area, favor the distribution of the substrate, and eliminate gaseous metabolites that can cause dead zones. The advantage of the pulsed-plate bioreactor with respect to the fluidized bed system is the lower energy demand. Biodegradation of phenol was investigated in a pulsed-plate column with the space between the plates packed with glass particles utilized as support for *Nocardia hydrocarbonoxydans* (Shetty et al. 2007a, b, 2011). These investigations mainly focused on evaluating the optimal operating conditions of frequency and amplitude of the pulsation (Shetty et al. 2007a) and dilution rate as a function of the influent phenol concentration (Shetty et al. 2007b).

Immobilized cell bioreactors

The immobilization of microorganisms into dedicated material is a common strategy employed for improving the removal of biorefractory and inhibitory compounds, and this approach has significant advantages in comparison to conventional suspended biomass systems. The first positive feature is the minimization of biomass losses as the cells are confined within a specific volume of the reactor. Moreover, the biomass concentration can be easily increased and the immobilization matrix can act, to some extent, as a protective barrier against the toxic effects of substrates (Li and Wang 2008, Juang and Kao 2009, Li and Loh 2007, Wang et al 2009). Immobilized cells can be utilized in fixed and fluidized bed reactors and in the recently described pulsed-bed bioreactor. Various support media for immobilization have been investigated, including membranes, alginate beads, sintered glass, gels, foams, GAC, but recent studies have mainly focused on membrane bioreactors.

*Membrane contactors*

Membrane bioreactors are commonly used for the treatment of both domestic and industrial wastewater. In their classical configuration, the membrane merely acts as a physical barrier to remove microorganisms and pollutants from the effluent. Thus, the membrane also provides a very efficient mean for retaining and actively controlling the biomass concentration inside the system in order to support a high volumetric removal capacity. These applications are

well described in the literature and will not be reviewed here. Instead, we will focus on recent applications utilizing the membrane's ability to offer a suitable support for microbial growth.

For instance, cells can be immobilized within the fibers of hollow-fiber membrane and/or grow attached onto the surface. This technology has been successfully used for treating high-strength phenol-laden streams and was recently proposed in applications utilizing *Pseudomonas putida* BCRC14365 (Juang et al. 2008; Juang and Kao 2009) with a polypropylene membrane and *P. putida* ATCC49451 (Li and Loh. 2007) with a polysulfone membrane. These studies aimed at evaluating the contribution of the immobilized biofilm on the biodegradation process (Juang and Kao 2009) and at minimizing the fouling caused by biofilm overgrowth on the shell side by adding the dispersing agent tetrasodium pyrophosphate (Juang et al. 2008). A fortuitous aspect of hollow-fiber membranes applied to phenol biodegradation in this study was the ability to deal with high-salinity water (Juang and Wu 2007) with a significant increase of the applicable influent load at NaCl concentration up to 1.52 M.

To limit toxicity effects without reducing the applicable influent load, Wang and Li (2007) developed an activated carbon-filled polyethersulfone composite hollow-fiber system used to immobilize *P. putida* for phenol removal. The incorporation of GAC enhanced the sorption capacity of the hollow fiber membranes but GAC bioregeneration was required for long-term operation in order to maintain high removal efficiency. This adsorptive deterioration was observed both during batch tests (Wang and Li 2007) and in a continuous system (Li and Wang 2008) where a progressive reduction of the removal efficiency was detected with increasing phenol loading. If bioregeneration is applied (i.e., regeneration of the activated carbon using the indigenous biomass after exhaustion of the phenol in solution), the required times could be very long (and not suitable for application) due to the strong adsorptive properties of GAC and the poor release of the adsorbed compound from the solid to the liquid phase.

An alternative configuration for membranes is the spiral module characterized by high membrane surface area per unit volume. Yordanova et al. (2009) investigated phenol biodegradation by *Aspergillus awamori* NRRL 3112 immobilized on a modified polyacrylonitrile membrane spirally wound in a lab-scale bioreactor with recirculation. Significant advantages in terms of stability and process efficiency were observed in comparison to the suspended cell system.

### *Gel immobilizing agents*

Calcium alginate beads have been extensively utilized as an immobilizing matrix and were recently applied to phenol biodegradation by *C. tropicalis* NCIM 3556 in a packed-bed column reactor (Varma and Gaikwad 2010). Calcium alginate is characterized by its high biocompatibility, low cost, easy availability, and simplicity of preparation (Shao et al. 2009) but can be subject to abrasion and gel deterioration as found by Ahamad and Kunhi (2011) who compared the performance of phenol degradation by *Pseudomonas* sp. CP4 cells entrapped in Ca alginate and agar gel beads in a fluidized bed reactor. Better efficiency was observed with the agar gel system that was able to degrade the highest phenol concentrations in the influent, up to 4,000 mg/L.

As an alternative to calcium alginate, El-Naas et al. (2009) proposed a polyvinyl alcohol (PVA) gel, a synthetic polymer possessing high mechanical resistance and greater durability. The PVA matrix was employed to immobilize cells of *P. putida* in a bubble column reactor for phenol removal and the effect of operating parameters and influent concentration toxicity on process performance were examined. The biodegradation rate was strongly affected by the substrate concentration with a pattern following Haldane kinetics (El-Naas et al. 2009). The PVA was also used in conjunction with *P. putida* for phenol biodegradation in a continuous spouted bed bioreactor (El-Naas et al. 2010). A cyclic motion of the particles within the bed was generated using a single air jet injected at the bottom of the reactor. The enhanced mixing reduced the effects of substrate inhibition.

### *GAC-composites as cell supports and substrate adsorbents*

Activated carbon has been used together with immobilizing agents PVA and xanthan gum (Kwon et al. 2009). The carbon content in the beads had to be limited to 1% because higher fractions decreased the mechanical strength of the beads. The composite polymer-activated carbon beads were then tested for phenol removal by immobilized *Pseudomonas fluorescence* KNU417 in a packed-bed bioreactor. The performance of microorganisms immobilized into PVA beads was compared to the performance of microorganisms immobilized into PVA-GAC beads. The beneficial effect of the activated carbon was limited to the start-up phase and similar removal efficiencies were recorded after stabilization. These data suggest (as it was observed in membrane and biofilm reactors) that the addition of activated carbon can be beneficial to prevent sudden shocks.

### *Foam matrices*

As an immobilizing agent, polyurethane foam contains macropores that provide low diffusional resistance and can efficiently support biomass growth. In a recent application,

Ribeiro de Nardi et al. (2007) investigated the biodegradation of BTEX by a bacterial consortium immobilized into polyurethane in a horizontal flow anaerobic immobilized biomass reactor. The authors reported BTEX removal efficiencies in the range of 41–77%. The same system was applied to the removal of BTEX by a denitrifying immobilized consortium by Ribeiro Gusmão et al. (2007), who observed the independent parallel removal of benzene, toluene, and xylene with high removal efficiencies (>90%). The advantages of cell immobilization onto polyurethane foam with respect to suspended biomass systems were also highlighted by Wang et al. (2009) in terms of improved performance and resistance to shock loadings for nitrobenzene removal. The immobilized cell reactor also supported higher removal efficiency in the presence of salinity, phenol, and aniline.

## Sequencing batch reactors

In SBRs, microorganisms are periodically exposed to cycling operating conditions. This substrate regime, to a certain extent, directs the composition and metabolic properties of the microbial cultures operating in the biological processes. This feature arises from the controlled, short term, unsteady state conditions that can favor the induction of enzymes able to develop specific metabolic pathways, and is of particular relevance in the biodegradation of biorefractory compounds (Venkata Mohan et al. 2005, Tomei et al. 2008). In addition, SBR reactors are characterized by high operational flexibility and a favorable cost-effectiveness ratio for small-scale treatment facilities (Sahinkaya and Dilek 2007).

Recent applications of SBRs have been reported for mixtures of substituted phenols. Tomei et al. (2008) investigated the kinetics of biodegradation of a 4-nitrophenol and 3,4-dimethylphenol in a conventional suspended biomass SBR finding an improvement in the degradation kinetics of the more slowly degradable compound (3,4-dimethylphenol) as a mixture in comparison with single compound tests. The kinetics of 4-chlorophenol and 2,4-dichlorophenol (which are supposedly more toxic than dimethylphenol) in batch and SBR reactors were investigated by Sahinkaya and Dilek (2007) and significant substrate inhibition was observed in single compound tests. In mixture, 4-chlorophenol degradation was strongly and competitively inhibited by the presence of 2,4-dichlorophenol. It was possible to reduce this effect by prolonging the feed times in the SBR. The inhibitory effect of 4-chlorophenol was also observed by Monsalvo et al. (2009) during the biodegradation of a mixture of phenol and 4-chlorophenol, and a beneficial effect was provided by an increase in temperature with more efficient degradation kinetics at 35°C.

To improve SBR operation during the treatment of wastewater containing inhibitory compounds, Moussavi et al. (2009) proposed a moving-bed sequencing batch reactor using mixed biomass attached onto cylindrically shaped particles of polystyrene. The objective was to combine the advantages of discontinuous operation with the performance of the moving bed biofilm reactors. The system was applied to a high-load phenolic wastewater and showed effective performance for influent phenol concentrations up to 3000 mg/L. A subsequent upgrade of this system, the moving bed sequential continuous inflow reactor (MSCR) was proposed by Moussavi and Heidarizad (2010). In the MSCR, the cyclic operational mode was maintained but the influent was added continuously at low flow rate. The system was applied to the biodegradation of a mixture of phenol, formaldehyde, and COD in wastewater, and removal efficiencies higher than 97% were obtained with the system showing a rapid recovery in the presence of hydraulic shock loads.

## Granular sludge reactors

Granular sludge is made of self-immobilizing cells and can be considered to be a special case of biofilm systems (Adav et al. 2008). Granulation was first applied to strictly anaerobic systems and later extended to aerobic systems in the late 1990s. Aerobic granules are mainly developed in SBR reactors and are densely packed microbial aggregates with densities much higher than that of activated sludge. Granules have excellent settling properties and can withstand high organic loads and toxic influents. The mechanisms of granulation are still poorly understood but experimental evidence suggests that accurate control of the hydraulics of the system is necessary to achieve the hydrodynamic shear force necessary to stabilize the three dimensional structure of the granules. Moreover, to achieve efficient operation, significant energy input (i.e., high recycle flows) is required to maintain a high degree of fluidization.

Recent applications of granular sludge targeted the treatment of high-strength phenol wastewaters. Ho et al. (2010) thus studied the inhibitory effect of high-phenol concentrations in batch tests and found the upper concentration limit for biodegradation inhibition to be >3,000 mg/L. Moussavi and Heidarizad (2010) utilized an aerobic granular SBR for the biodegradation of phenol in saline wastewater obtaining removal efficiencies ≥98% for influent concentrations in the range of 100–2,000 mg/L.

Granules degrading phenol were microbiologically analyzed by Adav et al. (2007), and accumulation of active biomass on the external layer was observed, from which *C. tropicalis* was isolated. The isolated strain was able to effectively degrade phenol and the inhibitory effect was detected at concentrations >1,000 mg/L.

Other applications of aerobic granules have been studied for the biodegradation of chlorinated phenols. Carucci et al (2010) investigated 4-chlorophenol removal in a granular SBR and reported very high removal efficiencies (99%) at an influent concentration of 50 mg/l. Wang et al. (2007) studied 2,4-dichlorophenol biodegradation in an aerobic granular SBR and reported a removal efficiency of 94% at an influent concentration of 105 mg/l.

### Evaluation of applicability

Many of the conventional configurations presented above have provided satisfactory treatment of monoaromatic substrates at the laboratory scale in terms of substrate removal efficiency. The practical implementation of these technologies, however, may not be entirely straightforward. Except for the configurations based on the use of adsorptive media (as supports or immobilizing agents), there is no physical reduction of the contaminant concentration and therefore the biomass, even if it experiences more favorable conditions in comparison to conventional suspended biomass reactors, is always exposed to high substrate levels. As a consequence, the effect of substrate toxicity can be attenuated but not completely eliminated. The operation of most of the bioprocesses describe here will also remain limited by the high risk of complete process failure (biomass loss) during episodes of toxic shocks. The addition of an adsorptive medium (generally activated carbon) can reduce the concentration level (at least temporarily) but generates the additional problem of producing a new polluted matrix that has to be treated or disposed of. Moreover, the immobilized cell bioreactors investigated utilized pure cultures, which will limit the likelihood of such systems being applied in practice, especially for wastewater treatment, which invariably involve mixed microbial populations. Studies aimed at the use of mixed population immobilized systems may, therefore, be a fruitful area of research.

Many of the conventional methods described required complex materials and equipment, and the need for accurate operational control relative to the most basic of systems (e.g., activated sludge) likely making these approaches not particularly robust and potentially quite expensive. These various technologies (e.g., membrane systems, immobilized cells) may therefore be economical only when used in bioprocesses that generate a saleable product (i.e., when *product* inhibition is ameliorated). Simple and inexpensive technologies are needed for the biological treatment of toxic pollutants. In the following, alternative technological solutions considering these prerequisites are presented and analyzed in terms of potential applicability.

## Two-phase partitioning bioreactors

Over the past 25 years, the concept of introducing a second immiscible phase into a bioreactor to enhance bioprocess performance has evolved from a rudimentary idea to numerous applications in biotechnology and environmental engineering. TPPB systems are all designed to address a common constraint of bioprocesses, namely, that the presence of toxic molecules in such systems can limit process performance. TPPBs, by means of an immiscible second phase, selectively partition toxic molecules either *to* the microorganisms in degradative reactions, or *away* from the microorganisms in synthesis reactions to eliminate toxicity and improve process performance. Below, we provide a brief historical perspective of the evolution of TPPB systems, describe the features and performance of three TPPB modifications (liquid–liquid, encapsulated, and solid–liquid) as applied to the biodegradation of monoaromatics and suggest future directions for this technology platform.

Biphasic systems were originally conceived to reduce end-product inhibition in the production of toxic fermentation products (e.g., ethanol, butanol) and, because immiscible organic solvents were used, this processing strategy (originally conceived in the 1980s) was called *Extractive Fermentation* (Kollerup and Daugulis 1985). By expanding the possible methods (e.g., pervaporation) available for removing inhibitory fermentation products directly from bioreactors, the term in situ product removal (ISPR) became a widely accepted expression for this strategy by the 1990s (Freeman et al. 1993). In the mid-1990s biphasic processing began to be applied to degradative systems in which toxic substrates were added to an immiscible organic solvent phase, to partition to a cell-containing aqueous phase based on cellular demand and on maintaining the thermodynamic equilibrium of the system. At this time, the term “Two Phase Partitioning Bioreactor” became the most widely used expression to describe such systems, and the use of this phrase has followed a very steep trajectory: from the ISI Web of Science database the first recorded use of the term “Partitioning Bioreactor” occurred in 1996, and as of the time of writing (December 2010), 195 articles have been published that use this phrase (35 articles in 2010), with 2061 citations in total (more than 500 in 2010). If “Partitioning Bioreactor” were a researcher s/he would have an h-index of 25. Research on TPPBs is being conducted in more than 20 countries around the world.

Although TPPBs continue to be effectively utilized to reduce inhibition in the production of toxic fermentation products (Gao and Daugulis 2009; Nielsen et al. 2010), TPPBs have overwhelmingly been applied to the treatment of inhibitory substrates, and this technology platform has been patented (Daugulis and Collins 2001). This review

focuses primarily on TPPB research published during the last 3–4 years in treating inhibitory monoaromatics, however, it is also striking to see the breadth of applications (i.e., to non-monoaromatics), and the large number of TPPB systems that have been studied over the past decade, as described in a recent excellent review (Quijano et al. 2009a). Although the motivation for these studies and those on monoaromatics that will be discussed in more detail here has, in most cases, been to reduce substrate toxicity, the use of a sequestering/delivery phase in TPPBs can also enhance substrate delivery of poorly water soluble compounds by providing a large substrate source for mass transfer, and can also positively influence the oxygen transfer rate.

## Liquid–liquid TPPBs

Immiscible organic liquids have overwhelmingly been the choice as the sequestering/partitioning phase in the biotreatment of toxic substrates in TPPBs (Quijano et al. 2009a). Silicone oil has been used in the vast majority of cases, with occasional selection of other organic solvents (e.g., bis-2-ethylhexyl sebacate and heptamethyl nonane (HMN)) and even ionic liquids (Baumann et al. 2005). Strategies for selecting TPPB solvents have been described in detail (Bruce and Daugulis 1991). Silicone oil (like HMN) has the desirable properties of being biocompatible with most microorganisms while also being essentially inert in terms of biodegradation (i.e., non-bioavailable), two features that are essential in biodegradative applications, particularly for mixed microbial populations. Notwithstanding the above potential advantages, silicone oil is far from ideal as a partitioning phase due to its high cost (circa €150–200/kg for the preferred low viscosity grade), high viscosity, tendency to cause foaming, adhesion to reactor internals and to biomass, and its potential to foul chromatography columns during analysis. Numerous other immiscible organic solvents have also been successfully used (Quijano et al. 2009a; Munoz et al. 2008), albeit often only with pure cultures to ensure non-biodegradability. Liquid polymer (Barton and Daugulis 1992) and cloud point systems (Wang et al. 2008) are two other liquid–liquid partitioning bioreactor systems that have been successfully demonstrated.

High concentrations of toxic monoaromatics have been successfully degraded in liquid–liquid TPPBs, in all cases at substantially superior rates than in single phase systems, due either to reduced toxicity achieved by the sequestering phase or enhanced substrate delivery (increased mass transfer) or both. Recent reports have included the degradation of pentachlorophenol (using dioctyl sebacate as the sequestering phase (Zilouei et al. 2008)), phenol (using kerosene (Juang and Tseng 2010; Juang et al. 2010)), benzene, toluene and phenol (using 2-undecanone (Hamed et al. 2004), toluene (using D-2-ethylhexyladipate (Darracq et al. 2010), 4-nitrophenol (using 2-undecanone (Tomei et al. 2008)), benzene (using *n*-hexadecane (Singh and Fulekar 2010), toluene (using *n*-hexadecane (Farhadian et al. 2010), and isopropylbenzene (using silicone oil (Aldric and Thonart 2008)). The quantitative improvements provided by TPPBs relative to single phase systems have been characterized in several ways: for example, feed benzene concentrations could be increased fivefold (880 to 4,400 mg/l) with similar performance (Hamed et al. 2004), degradation rate constants were increased threefold for the bioremediation of 4-nitrophenol in a TPPB relative to single phase operation (Tomei et al. 2008) and the volumetric removal rate for pentachlorophenol was increased by more than 100 times (1 to 142 mg/L h) via the use of a TPPB (Zilouei et al. 2008). In two instances (Darracq et al. 2010; Tomei et al. 2008), some degradation of the solvent delivery phase was observed, perhaps due to the fact that these systems used widely mixed populations of organisms, and again highlights one of the potential drawbacks of two-liquid phase systems for biotreatment applications. The potential biodegradability of the sequestering phase is less a problem in systems in which toxic fermentation products are removed via ISPR, because in such situations pure cultures are invariably used, and non-biodegradable solvents can usually be found.

An interesting operational consideration for liquid–liquid TPPBs is whether substrate mass transfer (from the solvent to the aqueous phase) or microbial kinetics is the rate limiting step in these systems. Two studies (Rehmann and Daugulis 2008b; Zilouei et al. 2008) have shown that mass transfer is not limiting, at least under the conditions studied, and this is likely due to the extensive dispersion of the solvent phase (and correspondingly small bubbles) in well-mixed bioreactors. A related practical matter is how the sequestering phase affects oxygen transfer, and a large number of studies have been conducted to examine the impact of the presence of an organic solvent on either $k_La$, or oxygen transfer rate (OTR). Enhancement of oxygen transfer has invariably been found for aqueous-organic TPPBs. In assessing such reports, it is important to make the distinction between these two metrics of oxygen transfer ($k_La$ and OTR) because, as has been discussed in earlier work on the subject (Nielsen et al. 2003; 2005), these two terms can sometimes be confused. Although in single aqueous phase systems, the $k_La$ and OTR have tended to be used almost interchangeably as an indicator of oxygen transfer efficiency (as they can when the oxygen driving force is fixed), lower $k_La$ values for TPPB systems do not imply a reduced OTR, merely that the system has taken longer to reach saturation due to the additional sink provided by the presence of an organic phase, whose oxygen solubility can be many fold higher than water.

Recent contributions in examining oxygen transfer in TPPBs include those by Quijano et al. 2009a, b; Torres-Martínez et al. 2010; and Quijano et al. 2010a, b, c. Liquid–liquid TPPBs have therefore not only been shown to detoxify numerous xenobiotic substrates, they also have the fortuitous and unforeseen benefit of enhancing the OTR in biotreatment applications.

In summary, liquid–liquid TPPBs have been shown to be superior to single-phase biotreatment systems due to their capacity to overcome substrate toxicity directly. The use of silicone oil will likely be the solvent of choice for many researchers; however, its use in bioremediation applications will probably be limited to academic studies, demonstrations of concept, and fundamental investigations (e.g., modeling, mass transfer) rather than full-scale implementation due to the high cost of this material, and the other limitations identified above. This is in contrast to ISPR applications of TPPBs (e.g., extractive fermentation) in which pure cultures are invariably used and alternative solvents (other than silicone oil) can usually be found. Moreover, the use of any immiscible organic liquid as the partitioning phase in commercial remediation applications may be impractical if direct contact between the solvent and the contaminated air, water, or soil site is required, due to the potential transfer of the solvent (via dissolution or adhesion) to the material being treated.

### Encapsulated liquid-phase TPPBs

In situations in which direct contact between an immiscible carrier phase and the degrading organism is undesirable (i.e., in cases in which the partitioning phase is biodegradable or cytotoxic), encapsulation of the solvent phase has been shown to function effectively. For example, liquid-core capsules composed of a dibutyl sebacate contained within a crosslinked alginate/polyacrylamide membrane showed reduced toxicity relative to single-phase biodegradation of atrazine (Wyss et al. 2006). Also, to reduce substrate and/or solvent toxicity, chitosan-coated PVA beads containing silicone oil were shown to enhance biodegradative performance (Sarma et al. 2010). Phenol degradation has also been demonstrated in a partitioning bioreactor in which the sequestering phase was either 1-nonanol (Zhao et al. 2010a) or modified montmorillonite encapsulated in polysulfone (Zhao et al. 2010b). In the latter case, phenol degradation was almost doubled (125.8 to 208.4 mg/L h) by operating as a TPPB rather than in single-phase mode.

The use of such encapsulation methodologies to separate a solvent from cells may have somewhat limited applicability in bioremediation processes, however. As noted above, TPPBs have been used for both biosynthetic applications (e.g., ISPR for toxic fermentation products) and biodegradative ones. In biosynthetic applications, there is greater flexibility in the selection of the partitioning phase, particularly considering its cost and/or complexity, since biosynthetic systems that produce a commercial product can generate a revenue stream. On the other hand, biotreatment processes are often driven by criteria such as simplicity and low cost, and in such cases it may not be feasible to use encapsulated complexes as the partitioning phase in TPPBs, particularly when simpler, and less expensive, alternatives (e.g., polymers) are available.

### Solid–liquid TPPBs

The first demonstration that solid polymer beads can replace immiscible organic solvents (Amsden et al. 2003) has spawned numerous subsequent studies of solid-liquid TPPBs successfully applied in both biosynthetic and bioremediation systems. The main advantages in substituting polymers for immiscible organic solvents are their completely inert nature (non-bioavailable and non-cytotoxic) and their exceedingly low cost. As an example, thermoplastic elastomers such as DuPont's Hytrel, cost less than €5 per kg. These features are extremely important in bioremediation, where mixed populations of organisms are invariably employed, and where costs need to be very tightly controlled. The fact that uptake by amorphous commercial polymers occurs by absorption rather than by adsorption confirms that the same mechanisms apply to solid–liquid TPPBs, namely, equilibrium uptake and release of toxic substrates, and delivery based on metabolic demand. The use of inexpensive, non-volatile, non-flammable, biocompatible and easily shaped polymers as the sequestering phase in a bioreactor is an enormous advance in designing high-efficiency and low-cost bioprocesses that eliminate cell toxicity and is key to the development of "green", solvent-free processing strategies.

Monoaromatics that have been biodegraded using polymer-based TPPBs include toluene (Daugulis and Boudreau 2008; He et al. 2009), phenolic mixtures (Prpich et al. 2006), BTEX compounds (Littlejohns and Daugulis 2008a), phenol (Amsden and Lau 2008), 4-nitrophenol (Tomei et al. 2009; Tomei et al. 2010), as well as hexane (Hernandez et al. 2010) and pinene (Montes et al. 2011). As expected, enhanced bioremediation performance was demonstrated in all cases relative to single-phase operation. By way of example, Tomei et al. (2010) found that a feed concentration of 1,000 mg/L 4-nitrophenol caused complete cessation of microbial growth due to substrate toxicity in single-phase operation, and that by adding only 5% (*v*/*v*) polymers and operating as a TPPB, the same reactor allowed rapid and complete biodegradation of this substrate. These authors also showed no long-term build up of substrate within the polymer matrix and

multiple re-use of the polymer beads with no decrease in performance of the sequestering phase. Selection criteria for the use of polymers have been formulated (Rehmann et al. 2007), and modification of the polymer to enhance performance through sonication (Isaza and Daugulis 2010) or through admixing polymers with magnetic beads (to facilitate removal of the sequestering phase from contaminated water and soil sites) has also been demonstrated (Yeom et al. 2010a). Polymeric wastes, such as shredded automobile tires have also been shown to work effectively as a sequestering phase in solid–liquid TPPBs (Prpich et al. 2008).

The enhancement of OTR, seen for two-liquid TPPBs, has also been confirmed for solid–liquid systems (Littlejohns and Daugulis 2007), making these systems similar in this aspect. Two liquid-phase systems do have an advantage over polymer-based TPPBs in substrate mass transfer (Littlejohns and Daugulis 2009; Hernandez et al. 2010), since some instances have been reported (Rehmann and Daugulis 2008c) in which the rate of substrate delivery from polymers was determined to be rate limiting; this could potentially be overcome with polymers of higher diffusivity or with small polymer beads with reduced diffusional path lengths. Given the past emphasis on the use of silicone oil in liquid–liquid TPPBs, it would be interesting to undertake a comparison between the use of this material in TPPBs with silicone rubber (Littlejohns and Daugulis 2007), a material which is identical in chemical composition, polydimethylsiloxane, but which is merely a (much cheaper) solid.

Although both liquid–liquid and solid–liquid TPPBs have shown significantly enhanced performance in all cases relative to single-phase systems, some assessment of the relative merits of these two types of TPPBs should perhaps be made. In terms of substrate detoxification, and hence reactor capability, liquid–liquid and solid–liquid TPPBs have demonstrated performance which in most cases has been indistinguishable (Boudreau and Daugulis 2006; Tomei et al. 2009), although solid–liquid TPPBs have been shown to be superior to liquid–liquid systems from an operability standpoint (Morrish and Daugulis 2008). Particularly in situations in which mixed cultures are used (certainly the case for practical bioremediation applications), two-liquid phase systems are limited to using perhaps only silicone oil or HMN. For this reason, the use of polymers has clear advantages as many thousands of commercial polymers are available with varying properties and affinities for target molecules, and the use of mixtures of polymers tailored to a particular mixture of target substrates is easily accomplished (Morrish and Daugulis 2008). From a practical standpoint, the use of polymers is also superior in terms of storage, handling of spills, safety, and recovery and reuse of the sequestering phase relative to the use of organic solvents. Critically, commercial polymers, including waste plastics and rubbers (such as automobile tires) will have the enormous advantage of being far less costly than solvents. In situations requiring direct contact with contaminated sources (e.g., contaminated soil) polymers again would appear to be superior, as well as eliminating concerns about flammability, viscosity, and losses through adhesion to particles (e.g., soil) or bioreactor internals. Unless the pollutants are available in a near-pure form (e.g., as stored or stockpiled pesticides and banned chemicals) and can be added directly to a TPPB, contacting and completely recovering an immiscible solvent from a contaminated source would be very difficult to achieve in practice without significant solvent losses and contamination of the environment by the solvent. Remediation of actual contaminated air, water and soil environments by uptake of the target pollutant by direct contact with polymer beads, followed by destruction of the toxic substrate in a solid–liquid TPPB has previously been demonstrated (Prpich et al. 2006; Prpich et al. 2008; Rehmann et al 2008a; Rehmann and Daugulis 2008d; Yeom et al. 2010a).

In the recent review of TPPBs in environmental biotechnology, Munoz and co-workers provided several conclusions and prospects for real-world applications of TPPBs (Quijano et al. 2009a): (1) TPPBs, have demonstrated superior performance to conventional biological techniques, (2) The use of liquid-liquid TPPBs is constrained by foaming, high-viscosity and high cost of the sequestering phase, (3) Solid–liquid TPPBs can potentially and satisfactorily address these limitations. We would also suggest that the added advantages of direct application to contaminated sites, and the potential of tailoring polymers and/or using waste polymers provide a bright future for solid–liquid TPPBs in environmental applications.

## Overall assessment

The area of TPPBs has matured over the past 15 years to the point where general design strategies have been formulated to aid in specifying TPPB operating protocols (Yeom et al. 2010b; 2010c), modelling under steady-state and dynamic conditions has been performed for liquid–liquid TPPBs (Fazaelipoor 2007; Nielsen et al. 2007) as well as solid–liquid systems (Littlejohns et al. 2010), scale-up has been examined (Marques et al. 2010) and economics assessed (Mahanty et al. 2010). One of us (AJD) is currently undertaking the design and fabrication of new polymers with targeted properties for use in TPPB systems for both biodegradative and biosynthetic applications. The TPPB technology platform, particularly in solid–liquid mode, has enormous scientific and practical potential in biosynthetic as well as environmental applications.

## Integrated chemical-biological treatment

### Rationale and principle

Despite the significant improvements arising from microbial acclimatization or controlled substrate delivery, it is not always possible or economical to treat industrial wastewater biologically. For instance, the biodegradation of certain pollutants may require very long cell retention times (e.g., 40 days for xenobiotic degradation) due to competitive substrate uptake and slow growth at low substrate concentrations. This requirement often means poor sludge settleability and high oxygen demand during aerobic treatment. Another drawback is the potential instability caused by fluctuating influent properties, especially when process performance relies on a diverse microbial community containing sensitive microorganisms (e.g., methanogenic archaea during anaerobic treatment, bacterial nitrifiers during combined nitrogen–carbon removal, or algae during photosynthetic oxygenation in ponds). In these situations, a chemical pre-treatment step can serve to convert the recalcitrant and/or toxic pollutants into biodegradable and biocompatible products before biological treatment (Scott and Ollis 1995, 1996, 1997; Esplugas and Ollis 1997). This integrated approach is considered as more cost-efficient and environmentally friendly than full chemical treatment for several reasons: (1) chemical wastewater treatment often requires higher amounts of energy (e.g., UVC-irradiation, sonication, ozonation) and chemicals (e.g., Fe(II), $TiO_2$, $H_2O_2$, etc) than biological treatments processes, (2) chemical treatment often only partially mineralizes organic pollutants into products that *must* be further degraded (meaning integration is often a de facto necessity), and (3) because of the high energy and material consumption highlighted above, it has been argued that intensive chemical treatment can have an overall negative impact on the environment due to the associated release of greenhouse gases to the atmosphere (Jones et al. 2007).

### Design and optimization

The design of an integrated chemical–biological process is generally based on the assumption that the chemical process is more costly and must be applied only until a significant enhancement in biodegradability is achieved. Under this assumption, an integrated process is optimized by determining the minimum chemical dose (e.g., UV, $H_2O_2$, or ozone dose) needed to allow subsequent biological treatment with acclimatized microorganisms under, possibly, substrate dilution in a continuous process. In the case of biorecalcitrant pollutants, the minimum dose is typically the dose that allows pollutant removal below targeted values for discharge (Zapata et al. 2010b; Mendoza-Marín et al. 2010). In the case of toxic pollutants, the dose depends on the process and microorganisms used for biological treatment (Essam et al. 2007b).

A mathematic approach based on mass balance analysis and experimental kinetic data was utilized to co-optimize phenol removal in an integrated UV-$H_2O_2$/biological process (Edalatmanesh et al. 2008). Unfortunately, the constructed model was not experimentally validated and has little predictive power in real applications because the mechanisms and rates of the chemical and biological reactions involved in the removal of organic pollutants are significantly affected by environmental conditions, wastewater composition, and scale effects. For instance, Essam et al. (2006a) reported that the simulated solar irradiation of a mixture of chlorophenols yielded toxic and biorecalcitrant products that were not detected when the pollutants were irradiated individually. Zapata et al. (2010a) observed that the irradiation time necessary to completely remove the aromatic pesticides imidacloprid and pyrimethanil from simulated wastewater by solar photo-Fenton treatment increased threefold when the photoreactor volume was scaled-up from 75 to 1,060 L, mainly due to wastewater salinity and temperature effects. Using the same process, Zapata et al. (2010b) also reported differences in the treatment efficiencies when a real wastewater was used instead of a simulated wastewater containing aromatic pesticides.

Because of the difficulty in predicting the efficiency of partial chemical treatments and the toxicity and biodegradability of the products formed, systematic experimental optimization remains necessary. For this purpose, Sarria et al. (2002) proposed to initially assess the wastewater toxicity and inherent biodegradability at various dilutions in order to determine if direct biological treatment of diluted substrate with acclimatized microorganisms is feasible. If this could not be accomplished, a pre-treatment would then be applied until significant toxicity reduction and/or biodegradability enhancement is achieved. Practically, such an optimization scheme requires the ability to monitor removal efficiency, toxicity, and biodegradability. “Generic” parameters such as the dissolved organic carbon concentration (DOC) and the chemical oxygen demand (COD) are therefore commonly used to evaluate removal efficiency and track potential degradation products. From these parameters, the average oxidation state (AOS) of the organic carbon in solution can also be calculated as:

$$\mathrm{AOS} = \frac{4(\mathrm{DOC} - \mathrm{COD})}{\mathrm{TOC}}$$

where the COD and TOC are expressed in molar concentrations (the AOS varies from −4 for $CH_4$ to +4 for $CO_2$). Most aromatic pollutants undergo sequential oxidation

reactions during chemical treatments and each reaction converts its reactants into more oxidized products. Consequently, as the pollutant concentrations, the DOC, and the COD decrease, the AOS typically increases during chemical treatment and becomes an indicator of the amount of degradation products formed and their oxidation state. Interestingly, the AOS also tends to correlate positively with the biodegradability of organic compounds. This correlation is explained by the fact that the chemical oxidation of aromatic pollutants often induces structural changes such as ring opening, hydroxylation, or dehalogenation (Suarez-Ojeda et al. 2007; Goi et al. 2004, Essam et al. 2007b) that also increase biodegradability (Goi et al. 2004, Loonen et al. 1999). Unfortunately, the same reactions can also cause the formation of toxic or biorecalcitrant compounds, explaining why the AOS cannot be used as a substitute for bioassays. For instance, Mehrvar and Tabrizi (2006) reported that UV–$H_2O_2$ treatment of alkylbenzene sulfonate actually decreased the biodegradability of the effluent in a sequencing batch reactor inoculated with activated sludge microflora. Likewise, Olmez-Hanci et al. 2010 reported that UV–$H_2O_2$ treatment of a simulated wastewater containing diethyl phthalate caused pollutant removal but no detoxification to activated sludge.

In order to provide the best conditions possible for microbial degradation to take place (e.g., microbial acclimation and substrate dilution), inherent biodegradation assays (e.g., 28 days Zahn–Wellens assays) are often preferred during the testing of integrated treatments. The BOD/COD and BOD/TOC ratios are also commonly used as indicators of biodegradability, with a $BOD_5/COD>0.4$ being considered as typical of easily biodegradable organic matter (Scott and Ollis 1997). Considerable care should however be taken when using the $BOD_5$ assay (no time is given for acclimation to occur), especially if biorecalcitrant compounds are found together with biodegradable compounds present at much higher concentrations.

Alternative monitoring strategies can be used in specific applications. For instance, Essam et al. (2006a, 2007a) used a mass balance analysis based on chloride and COD concentrations in order to determine the dechlorination efficiency of solar-based pre-treatment and the level of chlorination of the degradation products formed from chlorophenols. Because the toxicity and aerobic biodegradability of chlorinated aromatics is inversely correlated to the number of chlorine atoms each compound contains (see cited studies and references), the pre-treatment yielding the lower amount of chlorinated products (UV–$H_2O_2$–$TiO_2$) also yielded the higher amount biodegradable products and overall highest efficiency.

Numerous bioassays have been used to monitor toxicity during integrated chemical–biological treatment, *Vibrio fischeri* bioluminescence and activated sludge respirometry being the most popular. However, many authors have repeatedly failed to demonstrate toxicity reduction during chemical treatment using the *V. fischeri* toxicity assay, which can easily be explained by the fact that this marine bacterium is naturally poorly equipped to resist a demanding freshwater environment. This discussion is however rather irrelevant as different toxicity assays should be used with different purposes; activated sludge respirometry would logically be more suitable to determine inhibition risks during biological treatment with activated sludge microorganisms whereas a more sensitive assay should be used to assess the overall detoxification efficiency of the process with regard to the recipient ecosystem used for wastewater discharge. Finding the optimum dose for chemical treatment can however be difficult because risk prevention during the treatment of unpredictable or variable wastewater composition could require overdosing the chemical treatment (Zapata et al. 2010b), whereas overdosing may also cause toxicity to increase (Zapata et al. 2010a).

## Recent progress

Progress in the development of integrated chemical-biological processes were extensively reviewed by Scott and Ollis 1997 (Scott and Ollis 1997) and more recently by Mantzavinos and Psillakis (2004) and Tabrizi and Mehrvar (2004). Since these reviews, notable progress has been made in process scale-up, real wastewater treatment, and further demonstration for numerous types of pollutants and wastewater. For instance, Malato et al. (2007) demonstrated the removal of biorecalcitrant $\alpha$-methylphenylglycine at pilot scale using integrated solar-Fenton/biological treatment with immobilized biomass whereas Zapata et al. (2010a, b), as described above, demonstrated the removal of aromatic pesticides from simulated and real wastewater at various scales using the same process. Pilot-scale photo-Fenton treatment was thus able to significantly reduce the toxicity of an artificial mixture of five pesticides (two aromatics) to *V. fischeri* and activated sludge while increasing its inherent biodegradability from 50% to 95%. Overall, the integrated treatment supported a DOC removal of 84% at industrial scale, with 35% corresponding to the chemical step. Using the same process, Mendoza-Marín et al. (2010) reported that solar photo-Fenton treatment of simulated and real agro-industrial wastewaters containing the aromatic pesticides dichlorophenoxyacetic acid and diuron increased the effluent $BOD_5/COD$ from 0.3 to 0.6, thereby allowing an overall DOC removal efficiency of 82.5% after biological treatment (50% due to the solar photo-Fenton process). Vilar et al. (2011) reported that solar photo-Fenton treatment of a landfill leachate containing

caffeic acid (286–579 mg/l) increased the effluent $BOD_5$/COD ratio from 0.16 to 0.37 and its inherent biodegradability from 44 to 49% up to 89% depending on the $H_2O_2$ dose used. Salles et al. (2010) showed the electrochemical pre-treatment of aromatic pesticide Phosmet reduced toxicity to *V. fischeri* and increased biodegradability ($BOD_5$/COD ratio), thereby allowing subsequent treatment with activated sludge microflora (total mineralization of 97%). Suarez-Ojeda et al. (2007) reported near complete COD removal during the treatment of a high-strength *o*-cresol laden wastewater using an integrated catalytic wet air oxidation (with activated carbon as catalyst/biological treatment (activated sludge) process (the pre-treated wastewater made 30% of the effluent fed to the biological reactor).

## Overall assessment

The evidence reported herein and the information provided in earlier reviews show that the efficiency of integrated treatment has been validated for a wide range of pollutants and process configurations, including at industrial scale and using real effluents. However, research in the area still overwhelmingly focuses on the use of advanced oxidation processes combined with aerobic biological treatment. This is rather surprising considering that the greatest advantages of integrated treatment could be found when chemical pre-treatment are combined with "sensitive" biological processes in a more holistic approach aiming not only to remove a pollutant, but also reduce aeration costs, conserve energy, minimize sludge production, or combine nutrient and carbon removal; in other words, integrated treatments have hitherto been considered for hazardous pollutant removal only, regardless of the other issues faced during wastewater treatment. For instance, Essam et al. (2006b, c; 2007b) showed that chemical detoxification of phenolics-laden wastewaters was critical to allow subsequent biological treatment using a consortium of algae and bacteria. This fully solar-powered integrated approach improves energy efficiency during detoxification due to photosynthetic aeration as well as nutrient removal due to nitrogen assimilation by microalgae (Muñoz and Guieysse 2006). There is also a lack of studies comparing the efficiency of various integrated treatments for the same application. Such comparisons would be interesting as the integration of certain processes can be more difficult and costly due the requirements of water conditioning prior to biological treatment (e.g., pH neutralization after photo-Fenton and Fenton treatments, cooling after wet oxidation). For example, Essam et al. (2007a) compared the efficiencies of various simulated solar UV, UV–$H_2O_2$, UV–$TiO_2$, and UV–$TiO_2$–$H_2O_2$ pre-treatments for chlorophenol removal, and found that the type of chemical treatment used has a profound influence on the toxicity and biodegradability of the degradation products formed. Finally, there is a lack of chronic toxicity monitoring during integrated treatment. This is rather critical as various chemical treatments have been shown to produce such type of toxicants.

## Conclusions

Improving the design and operation of biological treatment processes for monoaromatics in real-life applications presents many challenges, including working within the following constraints: substrate toxicity; mixed populations of organisms; the need for robust operation in the face of dynamic conditions of feed composition, concentration and environmental parameters such as temperature; low cost operation.

Single-substrate and single-organism studies are useful in characterizing process fundamentals such as kinetics, but do not fully meet all of the challenges required for practical implementation. Process designs that provide potential operational improvements such as higher biomass levels (i.e., biofilm reactors) or protection from adverse conditions (cell entrapment) are also useful contributions but do not completely resolve the fundamental limiting characteristic of monoaromatics, which is cytotoxicity. Based on our review, it appears that solid–liquid TPPBs and integrated physical/chemical systems, which can potentially address all of the above constraints, are the most promising approaches for the biological treatment of monoaromatics. In spite of their operational success, however, neither of these approaches has yet been applied commercially at full scale and a number of research opportunities still exist.

For example, in the case of TPPBs, the implementation of effective and low-cost materials with appropriate transport properties such as high diffusivity and the design of contacting shapes such as beads, plates and films within an industrial context (mechanically agitated, airlift, packed bed, sequencing batch, etc.) has not been fully explored. In the case of combined treatment strategies, the matching of several available physical treatments of a particular contaminant stream to a biological one, and the assessment of toxicities also require additional research to reduce treatment costs and risks. We would also argue that current research focuses almost exclusively on substrate removal without taking into account the entire spectrum of process considerations (e.g., nutrient removal, sludge disposal) and sustainability issues (e.g., energy efficiency, green house gases emissions) that must now increasingly be considered when determining best practice. Nevertheless, the performance of these approaches to date in biologically treating

recalcitrant compounds such as monoaromatics is exceedingly promising.

**Acknowledgment** AJD is grateful to Timothy Simon for helpful discussions.

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