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Industrial wastewater treatment through bioaugmentation

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Abstract

Bioaugmentation of activated sludge processes through the addition of microorganisms is employed with the aim of enhancing treatment, in particular the removal of priority pollutants. With industrial wastewaters, studies have covered target pollutants including ammonia and polycyclic aromatic hydrocarbons (PAHs): compounds that are regulated around the globe. However, bioaugmentation is a technique that has been associated with doubt in regard to its ability to benefit treatment processes. Failure of bioaugmentation has been reported to be associated with numerous factors that include the growth rate being lower than the rate of washout, insufficient inoculum size and substrate availability. Limitations of bioaugmentation can be overcome through techniques that include increased inocula dosing, pre-acclimatisation of inocula in side-stream reactors, addition of nutrients and surfactants and application of sufficient acclimatisation periods. Surveys of the literature show that a key area for further research should be towards acquiring a better understanding of the degradation pathways where bioaugmentation is applied. There also remains a need to undertake bioaugmentation efficacy studies at full scale with test and control streams. Further reporting on the economic viability of the technique is also necessary.

Keywords: Bioaugmentation; industrial wastewater; nitrogen; polycyclic aromatic hydrocarbons; phenol

1. Introduction

Industrial wastewaters account for a large proportion of pollution in freshwater systems and are therefore regulated across the globe. For example, in Europe, industrial wastewaters are regulated under the Industrial Emissions Directive (IED) whilst in the United States they are regulated under the Clean Water Act (European Commission, 2015b; US.EPA, 2015). Under the IED, the compounds included in the regulation vary for each industrial process and are reported along with the associated emission limits in the best available techniques reference document (BREF) (European Commission, 2011). An activated sludge process (ASP) has been identified as the best available technique (BAT) to meet the required emission limits (**Table 1**) for a number of such wastewaters. This includes wastewaters from the milk and food industry, waste gas treatment, refinery of mineral oil and gas, iron and steel coke processing and glass manufacturing (European Commission, 2003, 2006, 2012, 2013a, 2013b, 2014, 2015a).

The suspended microbial mass in an ASP is responsible for the biodegradation of organic compounds via the metabolic reactions of the bacteria (Zhang et al., 2014a). Many industrial wastewaters contain a mixture of compounds that are recalcitrant and others that may be toxic; such wastewaters therefore have the potential to persist in effluents after an ASP. It is thus necessary to establish treatment methods which can cope with the complex mixture of compounds typically associated with industrial wastewaters. Bioaugmentation, the addition of supplementary microorganisms with their associated biodegradation capacities, may allow for the improved performance of ASPs (Semrany *et al*., 2012).

Table 1: Industrial Emission Directive emission limits for wastewaters for which an activated sludge process is recognised as the best available technique.

Wastewater origin	BAT emission limit (mg/L)	Reference
Produced Water (Oil and gas wastewater)	Hydrocarbon oil index: 0.1 – 2.5 COD: 30 – 125 TN: 1 -25	(European Commission, 2014)
Food and Milk: e.g. Raw dairy, Cheese, Mixed dairy, palm oil mill effluent.	Oil and grease: < 10 COD: <125 BOD_5: <25 TN: < 10 TP: 0.4-5	(European Commission, 2006)
Glass manufacturing	COD: < 5-130 Total hydrocarbons: <15 Ammonia (as NH_4): < 10 Phenol: < 1	(European Commission, 2012)
Coke making wastewater:	COD: < 220 BOD_5: <20 SCN: < 4 PAHs*: 0.05 Phenols: 0.5 TN: <15-50	(European Commission, 2013a)

*Sum of fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene and benzo[g,h,i]perylene

Industrial wastewaters represent some of the most challenging waters requiring treatment and therefore offer insight into some of the more complex situations in which bioaugmentation may be implemented. Benefits may include more stable operating conditions, better flocculation characteristics, decreased start-up times, resistance to shock loads and better cold weather performance (Stephenson and Stephenson, 1992; Van Limbergen, Top and Verstraete, 1998; Guo *et al*., 2009; Bartrolí, Carrera and Pérez, 2011; Qu *et al*., 2011). Bioaugmentation has been reported to be unpredictable (Boon *et al*., 2000), however, a number of factors have been highlighted as impacting successful bioaugmentation including: strain selection, addition and maintenance techniques and knowledge of the molecular biology and the capabilities of commercial products (Stephenson and Stephenson, 1992; Van Limbergen, Top and Verstraete, 1998; Thompson *et al*., 2005; Herrero and Stuckey, 2014).

2. Strain selection

The selection of a suitable strain is essential to the success of bioaugmentation. The selected strain(s) must be able to withstand the environmental conditions imposed on them within a treatment process including; temperature, pH, dissolved oxygen, nutrient availability, toxicity and microbial pressures (Bitton, 2011). It is well recognised that, without an understanding of the conditions within the treatment process, bioaugmentation is likely to fail due to the poor survival of the inoculum and/or competition from indigenous microbial populations (Stephenson and Stephenson, 1992; More *et al*., 2010). The selection and isolation of a strain from the indigenous population has become, progressively, the favoured approach as this increases the likelihood of success as the strain is already adapted to survival in the selected environment (Ueno *et al*., 2007). This approach can be taken when a species is present in a treatment process but in insufficient numbers for adequate treatment. Selection of a strain from an alternative site may be the only option when a compound cannot be degraded by the species already present at location, however, success may be limited if the environmental conditions are not conducive to the survival of the inoculated strain (Thompson *et al*., 2005).

87 Applications may include the use of a single strain or a combination of strains to produce a
88 suitable consortium (Khehra *et al*., 2005; Qu *et al*., 2011). An individual strain may be
89 selected for its ability to degrade a specific compound or due its role in a more complex
90 degradation pathway. A number of strains may be used to replicate a natural community,
91 enhance or replicate a catabolic pathway with numerous stages and/or degrade a number of
92 target pollutants within the same wastewater (Van Limbergen, Top and Verstraete, 1998;
93 Thompson *et al*., 2005). Increasingly, consortia are selected for bioaugmentation
94 applications, with degradation processes frequently built upon the combined actions of
95 numerous species, especially for the degradation of complex xenobiotic compounds (Stroo,
96 Leeson and Herb Ward, 2013).

97

98 The success of a consortium was demonstrated by Khehra *et al*. (2005) for the treatment of
99 recalcitrant dyes released from the textile processing industry. In laboratory investigations,
100 both single strains and the consortium were supplemented with 20 mg/L of dye. Whilst the
101 individual strains were able to decolourise 3 of the 6 dyes, to varying degrees, the consortium
102 decolourised of all of the dyes. Further to this, the time required for the decolourisation was
103 reduced from 24 hours to 8 hours. Due to the structural diversity of the dyes, the synergistic
104 actions of the consortium proved to have a beneficial role. Similarly, the synergistic actions
105 of a consortium previously developed by Chhatre *et al*. (1996) were recognised as important
106 by Domde, Kapley and Purohit (2007) in the treatment of petroleum wastewater. In this
107 application, a combination of isolates worked together to solubilise and then degrade various
108 components of crude oil. One isolate was responsible for producing a biosurfactant followed
109 by the emulsification of the crude oil which then made long chain aliphatics and aromatics
110 available to the other two isolates for degradation. This combination of isolates resulted in an
111 overall degradation rate of 65-70% and an increase in chemical oxygen demand (COD)
112 removal from 15% without bioaugmentation to 52.2% with the consortium (Chhatre *et al*.,
113 1996; Domde, Kapley and Purohit, 2007).

114

115 Genetic manipulation provides further opportunities for the degradation of compounds for
116 which a pollutant-degrading natural strain does not exist (Stroo, Leeson and Herb Ward,

2013). Microorganisms can be genetically engineered to over-express degradation genes or to exhibit increased survivability (McClure, Fry and Weightman, 1991; Nüβlein *et al*., 1992; Stroo, Leeson and Herb Ward, 2013). Such techniques enable the possibility of designing microorganisms to assist with the treatment of pollutants which require numerous degradation steps or those required to degrade xenobiotic compounds. Knowledge of the degradation pathways involved for such compounds is limited and a naturally occurring species capable of such degradation may not exist (Stroo, Leeson and Herb Ward, 2013). Microorganisms which have been genetically modified have been investigated in groundwater aquifer microcosms (Jain *et al*., 1987), lake waters (Awong, Bitton and Chaudhryt, 1990) and ASP (McClure, Weightman and Fry, 1989; McClure, Fry and Weightman, 1991). McClure, Weightman and Fry (1989) demonstrated that genetically engineered bacteria were able to survive within a laboratory-scale ASP and did not encourage protozoa reproduction despite large numbers of bacteria being inoculated. Additionally Nüβlein *et al*. (1992) were able to confirm that microorganisms that were genetically engineered were not only capable of surviving in an ASP but were also able to maintain their genetic information and degrade the required pollutants. Such genetic adaptation may allow for the design of microorganisms which are able to assist in the degradation of pollutants which require numerous degradation steps. Further to the genetic modification of microorganisms, gene bioaugmentation, which involves the use of catabolic mobile genetic elements (MGEs), has been highlighted in regard to its applicability to bioaugmentation (Stroo, Leeson and Herb Ward, 2013). Mobile genetic elements consist of pieces of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) which can be transferred from one organism to another (Stroo, Leeson and Herb Ward, 2013).

Despite the numerous possible ways in which genetic engineering may improve the future of bioaugmentation, current research is heavily laboratory based and success in the field cannot currently be fully assessed due to legislative restrictions resulting from concerns surrounding the risks to both the environment and human health of the uncontrolled spread of microorganisms which have been genetically engineered (Van Limbergen, Top and Verstraete, 1998). Strategies such as the use of a ‘suicide element’ and immobilisation techniques have been considered in order to reduce such risks (Liu, Huang and Wang, 2008;

148 Stroo, Leeson and Herb Ward, 2013). Suicide techniques, for example, may be repressed by
149 an environmental signal such as from the pollutant to be degraded. When the signal no longer
150 exists the suicide element is activated. This technique has been shown to be successful in
151 preventing the spread of engineered cells (Torres, Garcia and Diaz, 2003). Legislation often
152 ignores the ways in which molecular genetics can be used for risk mitigation, and
153 consequently, future research will have to both inform and follow the regulations (Davison,
154 2005; Stroo, Leeson and Herb Ward, 2013).

155

156 Commercial inocula are now also widely available. Such products vary greatly in their make-
157 up, cell density, advised dosing rates and the incorporation of other additives e.g. stabilisers
158 and nutrients. Each of these factors need to be considered when selecting a suitable product
159 (Stroo, Leeson and Herb Ward, 2013). The use of commercial inocula may offer a short-term
160 solution to an immediate treatment issue; however, success rates may vary because such
161 inocula are typically produced and tested under stable conditions. Such conditions do not
162 reflect the real-life scenario relevant to many industrial wastewaters, in turn reducing the
163 survivability of the inocula (Stephenson and Stephenson, 1992).

164

165 **3. Operational considerations**

166

167 The application of bioaugmentation is more likely to be successful in a treatment system with
168 well-characterised wastewater and operational parameters. This knowledge helps to identify
169 potential obstacles to the survival of the inoculated bacteria, including toxicity and nutrient
170 availability (Jianlong *et al*., 2002). Without a detailed knowledge of the system, the
171 likelihood of a successful integration of the inoculum is reduced. Activated sludge processes
172 can differ greatly in their configuration, although principally they consist of one or more
173 treatment cells containing biomass which may be aerobic, anoxic or anaerobic in nature. Such
174 treatment cells may operate under continuous flow conditions, in mixed systems or be
175 operated under a batch or plug-flow system. The introduction and maintenance method for
176 bioaugmentation applications should therefore be informed by the design of the treatment

system. Treatment efficiencies and pollutant concentrations, on the other hand, will inform decisions relating to dosing rates (Stephenson and Stephenson, 1992; Park *et al*., 2008).

3.1 Dosing technique

Direct dosing involves the addition of the selected microorganisms straight into a treatment vessel. Such a technique represents the simplest method of bioaugmentation and can be advantageous in the sense that it can be applied as and when required. This can also be economically beneficial as it does not require plant modification and the associated operational costs. Problematic to this approach, however, is the reduced survival rates of the inoculated microorganisms due to a lack of acclimatisation to the environmental conditions of the host environment resulting, for example, from toxicity, pH, carbon availability, predation and competition between the indigenous and inoculated bacteria (Chong, Pai and Chen, 1997; Bouchez *et al*., 2000; Songzhe *et al*., 2009). The use of a side-stream reactor can help to overcome some of the aforementioned problems as it enables the acclimatisation of the inoculated microorganisms to the environmental conditions, thus increasing their survival rate in the treatment process (Parker and Wanner, 2007). The footprint of a side-stream is typically approximately 10% that of the main reactor. As the side-stream can enable process intensification, this can represent a much smaller investment cost than the cost associated with expanding a treatment works to cope with a higher capacity. Despite this, in some instances the additional land requirements may still be problematic (Salem *et al*., 2003). The use of encapsulation techniques can assist in the incorporation of inoculated bacteria into the existing flocs (Stormo and Crawford, 1992). Bouchez *et al*. (2009) mixed the inoculum with an alginate solution, forming bead structures which were employed in the reactor. The beads allowed the inoculated bacteria to remain in the system and protected them from the intense grazing that was experienced without such encapsulation. The beads were observed to break into fragments by day 8, but such fragments were incorporated into flocs of the indigenous sludge, allowing their successful incorporation into the system. Another recent strategy that has been showed successful treating high strength pyridine wastewater is through the addition

206 of aerobic granules of pure strains formed in a sequencing batch reactor (SBR) to the main
207 treatment reactor (Shen *et al*, 2009; Liu *et al*, 2015).

208

209 **3.2 Dosing location**

210

211 The success of bioaugmentation has been shown to be influenced significantly by the location
212 at which the selected microorganisms are dosed. Dosing location should be selected based on
213 a careful consideration of the environmental conditions that the selected microorganisms
214 require in comparison to those they will face. Determination of the most suitable location
215 may be more critical in industrial wastewaters, which frequently contain single or multiple
216 pollutants known for their toxic effects. The impact of the identification of the correct
217 location was demonstrated by Jianlong *et al*. (2002) during the treatment of coke-making
218 wastewater. The wastewater, characterised by the presence of multiple toxic compounds, was
219 treated through an ASP with an anaerobic, anoxic and aerobic reactor. *Burkholdiera pickettii*,
220 a quinoline degrading species, was shown to have a beneficial role at any location;
221 nevertheless, its positive impact was higher when *Burkholdiera pickettii* was added to the
222 aerobic reactor. The provision of a suitable food source and the lower toxicity, as a result of
223 the degradation of co-occurring compounds in previous treatment cells to smaller
224 compounds, resulted in higher degradation efficiencies. Similar conclusions were drawn for
225 the removal of 2-4-dichlorophenol in a laboratory-scale ASP. A mixed culture was developed
226 through the enrichment of sludge taken from two wastewater treatment plants. The mixed
227 culture was then added to a separate reactor with a carrier system of plastic lace strings
228 (Quan, Shi, Liu, Wang, *et al*., 2004). Removal was higher, at 90.3%, when the bioreactor was
229 located after the aeration cell than when the bioreactor was situated before the aeration cell
230 (86.2% removal). It had been assumed that locating the bioreactor before the aeration cell
231 would allow the removal of 2-4-dichlorophenol, which in turn would improve the removal
232 efficiency of other pollutants as a result of the lowered toxicity of the wastewater. Despite
233 this, the 2-4-dichlorophenol removal decreased when the bioreactor was placed before the
234 aeration cell as a lack of easily degradable compounds resulted in a decrease in the removal
235 of the targeted 2-4-dichlorophenol. When the bioreactor was placed after the aeration cell, the

bioaugmented culture was able to specialise in the removal of 2-4-dichlorophenol, increasing the treatment efficiency.

3.3 Dosing size and regime

Dosing characteristics and regimes vary considerably between the different applications of bioaugmentation. The first characteristic that requires consideration is the initial inoculum size, which should be sufficiently large enough to overcome initial predation pressures whilst not so large as to result in a disturbance to the ecosystem equilibrium. Ramadan, El-Tayeb and Alexander (1990) reported that p-nitrophenol containing wastewater required a high initial dose (4.3×10^4 cells per mL) in order to overcome predation pressures. In contrast, Bouchez *et al*. (2000) reported a disturbance of the ecosystem balance resulting from increased pressures due to a large inocula dose Secondly, the use of maintenance doses may be necessary in order to maintain the population of the inoculated bacteria which may decrease over time as a result of routine sludge wastage or inherently low survival rates. The need for a maintenance dose varies from application to application. Boon *et al*. (2003) noted that bioaugmentation was not a permanent process when investigating the removal of 3-chloroaniline whilst Martín-Hernández, Suárez-Ojeda and Carrera (2012) reported that maintenance doses were not necessary when the initial dose was high enough to overcome initial predation pressures.

4. Bioaugmentation failures and associated improvement techniques

Successful reports concerning bioaugmentation have also been associated with reports of unsuccessful bioaugmentation attempts. Fundamental to the success of any application is the ability of the inoculated bacteria to survive and prosper. Numerous factors have been cited for the failure of bioaugmentation (**Table 2**) including the growth rate of the microorganism being lower than the rate of washout (Boon *et al*., 2000), an insufficient inoculum size (Ramadan, El-Tayeb and Alexander, 1990), an insufficient substrate (Goldstein, Mallory and

Alexander, 1985; Bouchez *et al*., 2000; Tyagi, da Fonseca and de Carvalho, 2011; Martín-Hernández, Suárez-Ojeda and Carrera, 2012), predation by protozoa (Goldstein, Mallory and Alexander, 1985; Boon *et al*., 2000; Bouchez *et al*., 2000), competition between the inoculated and indigenous bacteria (Stephenson and Stephenson, 1992; Bouchez *et al*., 2000; More *et al*., 2010), the presence of other inhibiting substances (Goldstein, Mallory and Alexander, 1985; Bouchez *et al*., 2000; Tyagi, da Fonseca and de Carvalho, 2011), the availability of alternative substrates (Goldstein, Mallory and Alexander, 1985; Chitra *et al*., 1995; Quan, Shi, Liu, Wang, *et al*., 2004; Mahin *et al*., 2011), the need for an acclimatisation period (Stephenson and Stephenson, 1992) and extremes in environmental factors such as temperature and pH (Tyagi, da Fonseca and de Carvalho, 2011). An understanding of the root cause of the failure of the bioaugmentation process is important to ensure the advancement of bioaugmentation applications.

Grazing was held responsible for the failure of *M*. *aerodenitrificans* becoming established in an aerobic nitrifying sequencing batch reactor by Bouchez *et al*. (2000). The added bacteria were associated with the liquid phase of the reactor and were not incorporated into bacterial flocs. As a result of their suspended nature they were targeted by grazing protozoa, which have grazing rates proportional to the fast rates of decline seen in the system. Ramadan, El-Tayeb and Alexander (1990) also saw a decline in the inoculated bacterial abundance which coincided with the multiplication of protozoa in the treatment of p-nitrophenol (PNP)-containing wastewaters. Similarly, an overgrowth of protozoa as a result of bioaugmentation was reported by Songzhe *et al*. (2009) during the removal of ammonia from marine aquaculture wastewaters. Furthermore, a rapid decline of the denitrifier *Pseudomonas stutzeri* TR2 was again associated with probable predation during the treatment of piggery wastewater (Ikeda-Ohtsubo, Miyahara, Kim, *et al*., 2013). Songzhe *et al*. (2009) concluded that a form of protection, e.g. encapsulation from grazing, was necessary. An alternative approach investigated related to the ability of heat treatment to protect the inoculated bacteria from predation (Ikeda-Ohtsubo, Miyahara, Yamada, *et al*., 2013) and results showed that adapting the reactor conditions overcame the predation problems. When the temperature of the treatment reactor was reduced to 35°C the predators were able to proliferate and during this period, there was a rapid tenfold increase in their associated genes. When the temperature

was increased to 40-44°C there was no increase in the number of genes representing predators and therefore *Pseudomonas stutzeri* TR2 was protected from predation.

Contrary to reports concerning the negative effects of grazing on bioaugmentation, Yu, Peng and Ren (2011) demonstrated that grazing did not have a significant impact on nitrogen removal. Nitrification efficiencies were monitored in a bioaugmentation system in which all protozoa were inhibited and compared to one in which protozoa were not inhibited. Although initially protozoa numbers increased rapidly in the non-inhibited reactor, their numbers then declined gradually over the duration of the study and complete nitrification was ultimately possible in both reactors. The increased time requirement, from 71 days with protozoa inhibition to 76 days without protozoa inhibition, was not considered to be significant.

Nutrient limitation is a particularly important factor in the treatment of industrial wastewaters which frequently lack the essential nutrients required for microbial development (Burgess, Quarmby and Stephenson, 1999). Nutrient limitations have been held responsible for failed bioaugmentation attempts due to the competition between the indigenous and inoculated bacteria. Ramadan, El-Tayeb and Alexander (1990) demonstrated that the supplementation of nutrients could increase the likelihood of a successful bioaugmentation outcome as the addition of nitrogen and phosphate allowed for low densities of inoculum to remove p-nitrophenol (PNP), potentially increasing the inoculum growth rates and resistance to higher protozoa numbers. Such nutrient addition is referred to as biostimulation. Biostimulation, however, can also include the addition of other stimulants such as surfactants. Nikolopoulou, Pasadakis and Kalogerakis (2013) demonstrated that the presence of a biosurfactant could increase degradation rates in oil-contaminated sites by enhancing the solubility of the hydrocarbons. Under such treatment systems it is important, however, to have adequate controls in place in order to be able to assess to what degree the improvement is a result of the biostimulation rather than a result of the bioaugmentation itself. Due to its complementary action, biostimulation has therefore become a technique that is frequently reported for use alongside bioaugmentation (Wenderoth *et al*., 2003; Olaniran, Pillay and Pilay, 2006; Tyagi,

325 da Fonseca and de Carvalho, 2011; Nikolopoulou, Pasadakis and Kalogerakis, 2013; Shoji *et*
326 *al*., 2014; Sun *et al*., 2014).

327

328 Industrial wastewaters are frequently characterised by changing load rates which result in
329 fluctuating concentrations of the target compounds. Some failures of the bioaugmentation
330 process have been linked to long periods of starvation in the target pollutant. One means with
331 which to tackle this problem is to select an initial dose which is high enough to allow a
332 proportion of the bacteria to persist in the treatment system until the load rate increases again.
333 This approach was investigated by Martín-Hernández, Suárez-Ojeda and Carrera (2012)
334 during the treatment of p-nitrophenol in a laboratory-scale sequencing batch reactor. Using a
335 dose rates of 2% and 5% respectively, it was found that the higher initial dose rate allowed
336 the inoculated bacteria to survive the 20 day period of starvation and maintain subsequent
337 treatment. Importantly, the dose rate of 5% was still practical in terms of its application to
338 full-scale treatment works. In contrast Duque *et al*. (2011) found that periods of substrate
339 inhibition did not cause failure during the treatment of 2-fluorophenol in a rotating biological
340 contactor.

341

342 For some bioaugmentation applications failure lies in the inadequate adaptation of the
343 inoculum to the host environment. Chong, Pai and Chen (1997) reported that a mixed culture,
344 designed to treat petroleum wastewater, was unable to proliferate in the system, yielding no
345 benefit to the water treatment under pH shock conditions and complete failure under
346 continuous high pH conditions. The failure was linked to biomass washout as a result of
347 growth retardation or death of the inoculated population. Biomass washout, as a result of poor
348 reactor conditions, including an inadequate carrier system and violent air bubbling, was also
349 reported by Park *et al*. (2008) in the treatment of cyanide wastewater. Additionally, Songzhe
350 *et al*. (2009) reported that inoculated bacteria were unable to form an adequate biofilm due to
351 interaction with other indigenous bacteria resulting in biomass washout and the failure of
352 bioaugmentation. The likelihood of inadequate adaptation is increased with industrial
353 wastewaters and this highlights the requirement for understanding the treatment conditions
354 and adaptation techniques.

355 **Table 2: Reasons for bioaugmentation failures and possible improvement techniques.**

Problem	Technique to overcome problem
Predation (Overgrowth of protozoa) (Goldstein, Mallory and Alexander, 1985; Ramadan, El-Tayeb and Alexander, 1990; Songzhe *et al*., 2009; Martín-Hernández, Suárez-Ojeda and Carrera, 2012)	High initial doses (Ramadan, El-Tayeb and Alexander, 1990) Protection from grazing (Songzhe *et al*., 2009) Heat treatment (Ikeda-Ohtsubo, Miyahara, Yamada, *et al*., 2013)
Competition for nutrients between indigenous and inoculated bacteria (Ramadan, El-Tayeb and Alexander, 1990; Yu *et al*., 2005; Martín-Hernández, Suárez-Ojeda and Carrera, 2012)	Supplementation of nutrients (biostimulation) (Ramadan, El-Tayeb and Alexander, 1990)
Insufficient inoculations (Ramadan, El-Tayeb and Alexander, 1990)	Repeated inoculations (Boon *et al*., 2003) Continual inoculations (Abeysinghe *et al*., 2002)
Poor biofilm formation (Park *et al*., 2008; Songzhe *et al*., 2009)	Immobilisation/encapsulation (Stormo and Crawford, 1992; Quan, Shi, Liu, Lv, *et al*., 2004)
Wash-out (Chong, Pai and Chen, 1997; Park *et al*., 2008)	High initial doses (Ramadan, El-Tayeb and Alexander, 1990) Immobilisation/encapsulation (Stormo and Crawford, 1992; Quan, Shi, Liu, Lv, *et al*., 2004)
Decline of inoculated bacteria due to toxins (Goldstein, Mallory and Alexander, 1985)	Protection from adverse environmental conditions (Songzhe *et al*., 2009) Allow acclimatisation period (Stephenson and Stephenson, 1992) Use autochthonous bioaugmentation (Ueno *et al*., 2007)
Alternative substrates available (Goldstein, Mallory and Alexander, 1985; Chitra *et al*., 1995; Quan, Shi, Liu, Lv, *et al*., 2004; Mahin *et al*., 2011)	Detailed understanding of ecological background (Songzhe *et al*., 2009)
Large inoculations disturbing balance of ecosystem (Bouchez *et al*., 2000)	Careful consideration of dose rate
Periods of starvation (Martín-Hernández, Suárez-Ojeda and Carrera, 2012)	Higher dose rate to allow survival in the system for longer time periods (Martín-Hernández, Suárez-Ojeda and Carrera, 2012)

356

5. Applications of bioaugmentation to pollutants regulated by the Industrial Emissions Directive

A wide variety of wastewaters are regulated under the IED, all of which could potentially benefit from the application of bioaugmentation. An improved understanding of the capabilities of bioaugmentation could therefore offer widespread opportunities for industrial wastewater treatment. Industrial wastewaters can encompass a wide variety of pollutant compounds, although typically some commonalities exist between the different wastewaters. Nitrogen compounds are common to many types of wastewater, particularly those from the milk and food industries as well as coke processing activities. The levels of ammonia in coke-making wastewater can vary from 123 mg/L up to 4,500 mg/L (Ganczarczyk, 1983; Gould, 1986). Ammonia concentrations vary between sites due to variations in the operational conditions and also temporally at a single site due to variations in production levels (Marañón *et al*., 2008). High concentrations of ammonia are also characteristic of dairy wastewaters. As with coke-making wastewaters, they are subject to both spatial and temporal variations due to difference in the products produced and the treatment methods in place. Furthermore, these wastewaters are often produced intermittently (Vidal *et al*., 2000).

Nitrogen is a key target pollutant as it can cause the eutrophication of receiving waters. Nitrifying bacteria grow more slowly than the general heterotrophic community and are less resistant to toxicity. Consequently, nitrifying bacteria may be outcompeted. Supplementation through bioaugmentation may therefore be beneficial to treatment systems characterised by a high nitrogen loading. As the removal of nitrogen occurs in a two-step process involving the oxidation of ammonia to nitrite and the subsequent oxidation of nitrite to nitrate, nitrifying treatment processes require process stability in order to allow the two steps to remain synchronised and to prevent accumulation of the more toxic nitrogen species nitrite. Abeysinghe *et al*. (2002) investigated the ability of bioaugmentation to support the nitrification process when operating under stress conditions. At a solids retention time of two days, the treatment system operated near washout conditions, but the addition of 45 and 67.5 mg/L of ammonia oxidisers, allowed effluent ammonia concentrations to be reduced from 4.5 mg/L to <1 mg/L. The application of bioaugmentation can therefore be an effective and

388 convenient tool to support industrial treatment systems which frequently operate under stress
389 conditions.

390

391 Obbard and Shan (2001) also reported the use of bioaugmentation to support the treatment of
392 prawn aquaculture ponds which are characterised by high nitrogen loading rates but which
393 experience high levels of nitrifier washout as a result of the regular exchange of pond water
394 exchange employed to prevent the build-up of toxins in such ponds. Inert media have been
395 reported to enhance treatment by increasing bacterial populations through biofilm formation
396 (Stephenson *et al*., 2013). This technology has been selected in order to tackle the problem of
397 washout, with indigenous nitrifiers immobilised onto porous clay pellets, allowing the total
398 ammonical nitrogen to be reduced from 3 mg/L to 0.5 mg/L, the latter being below the
399 required concentration necessary for optimum prawn growth (1.33–1.53 mg/L) (**Table 3**).
400 The treatment of high nitrogen loads through bioaugmentation was reported by Onyia *et al*.
401 (2001) for palm oil wastewater (**Table 3**). Palm oil wastewaters are characterised by organic
402 nitrogen loads of 180–1,820 mg/L and the treatment of this type of wastewater is time
403 intensive, with more than 11 days required in order to achieve 50% nitrification. However,
404 the addition of 15 mg/L of a mixed nitrifying culture increased this efficiency to 100% within
405 seven days.

406

407 Carrier materials have also been employed to support bioaugmentation. In the treatment of
408 petrochemical wastewater, Ma *et al*. (2009) used a carrier system of polyurethane foam to
409 encourage the inoculated bacteria to form a biofilm (**Table 3**). The resulting biofilm
410 prevented the washout of the inoculated bacteria as well as the gradual decrease in their
411 numbers as a result of predation. Additionally, the inoculated bacteria were provided with
412 organic substrates and inorganic trace elements to support their growth. Consequently, the
413 bioaugmented reactor showed better performance with decreased start-up times (20 days
414 compared to 30 days without bioaugmentation), a higher resistance to shock loads of COD,
415 higher treatment efficiencies of refractory organic compounds (reduced to 21 compared to 46
416 without bioaugmentation) and a reduction of effluent ammonia concentrations (4.1 mg/L
417 compared to 12.4 mg/L).

418 **Table 3: Examples of bioaugmentation applied to compounds present in industrial**
419 **wastewaters.**

Compound	Scale	Application	Conclusions
Nitrogen			
(Onyia *et al*., 2001)	Laboratory	Palm oil effluent	15 mg/L of mixed cultures led to 100% increase in nitrification. Reduced HRTs led to 20% reduction in land requirement.
(Obbard and Shan, 2001)	Laboratory	Prawn aquaculture wastewaters	Immobilised bacteria allowed total ammonical nitrogen reduced from 3 mg/L to 0.5 mg/L allowing ponds to remain at optimal conditions.
(Ma *et al*., 2009)	Full-scale	Petrochemical wastewaters	Immobilisation prevented washout of nitrifiers. National discharge limits met in 20 days compared to 30 days. Effluent ammonia concentrations fell from 12.4 mg/L to 4.1 mg/L.
Aromatic compounds			
(Qu *et al*., 2011)	Laboratory	Synthetic alkaline wastewaters	Addition of Pseudomonas sp. JY-2 allowed improved start-up times (90% removal compared to 65% after 1.5 days) and increase long-term treatment efficiency (90% compared to 80%).
(Fang *et al*., 2013)	Laboratory	Coal gasification wastewater	Bioaugmentation increased removal efficiencies from 66% to 80% despite high variation in levels of phenol (500-3000 mg/L).
(Duque *et al*., 2011)	Laboratory	2-fluorophenol wastewaters	2-fluorophenol degrading species FP1 allowed treatment of waters subjected to shock loads of up to 50 mg/L of 2-fluorphenol.
(Martín-Hernández, Suárez-Ojeda and Carrera, 2012)	Laboratory	p-nitrophenol (PNP) wastewaters	Bioaugmentation allowed immediate removal of shock loads of PNP. Without bioaugmentation PNP removal took 4 days to reach 100% and then failed after 8 days.
(Straube *et al*., 2003)	Laboratory and pilot-	PAH contaminated soil	Bio-surfactant producer *Pseudomonas aeruginosa* strain 64 increased PAH

	scale		degradation from 23% to 34%. Bioaugmentation and biostimulation increased degradation to 87%. Biostimulation alone increased degradation to 86%.
(Sun *et al*., 2014)	Pilot	Former coke works contaminated soil	Total PAH levels fell by 24% in the control, 35.9% with bioaugmentation, and 59% with biostimulation. Bioaugmentation was responsible for the increased removal of heavy molecular weight molecules.

420

421 Bioaugmentation has also been used for the treatment of aromatic compounds including
422 phenols and polycyclic aromatic hydrocarbons (PAHs) which are present in a wide variety of
423 industrial wastewaters, including those from agrochemical, pharmaceutical, petrochemical,
424 coal gasification, coke processing, insecticide and hydrocarbon wastewaters among others
425 (**Table 3**). Aromatic compounds are regulated under the IED and are also listed as Priority
426 Substances within the European Union (European Union, 2013).

427

428 Coal gasification wastewater is subject to a high variability of phenol concentration, from
429 500–3,000 mg/L as a result of fluctuations in the pre-treatment performance. Such variability
430 can be problematic in regard to biological treatment due to the changing substrate levels and
431 the subsequent decline in bacterial numbers during periods of limited food supply. However,
432 system stability is of increasing importance as emission limits continue to be lowered. The
433 addition of phenol-degrading bacteria by Fang *et al*. (2013) (**Table 3**) allowed phenol
434 treatment efficiencies to increase from 66 to 80% and further increased the resistance to
435 fluctuating loads. Ammonia removal also improved (5 to 25%), although fluctuating
436 ammonia load rates required a higher recovery time. Resistance to shock loading of phenolic
437 compounds was also seen to improve due to bioaugmentation in the work of Duque *et al*.
438 (2011) for the removal of 2-fluorphenol. Interestingly, Duque *et al*. (2011) promoted biofilm
439 formation in a rotating biological contactor (RBC) through batch application of the inoculum.
440 This technique provided a means via which the system was able to stabilise and consequently
441 long-term maintenance was not required. This allowed for improved resistance to shock loads
442 and increased resistance to periods of starvation (**Table 3**). Although improved resistance to
443 shock loads of p-nitrophenol was also observed by Martín-Hernández, Suárez-Ojeda and

Carrera (2012), resistance to starvation periods was determined as a function of the size of the initial inoculum dose (**Table 2**).

The stable removal of both pyridine and quinoline from coke-making wastewater was observed after the inoculation of a laboratory-scale sequencing batch reactor filled with modified zeolite (Zhang *et al*., 2014b). Removal of both compounds was maintained at 100% whereas removal efficiencies could vary from 0 to 93% without bioaugmentation. This was attributed to an improved bacterial diversity, which increased the resistance to shock loadings. The interaction of species in a mixed culture of four species (*Paracoccus* sp. BW001, *Shinella zoogloeoides* BC026 and *Pseudomonas* sp. BC001) was believed to be responsible for the success of bioaugmentation for the removal of pyridine and quinoline in coke-making wastewaters (Bai *et al*., 2010).

Polycyclic aromatic hydrocarbons (PAHs) can be found in oil and gas wastewaters as well as coke-making wastewaters and are typically difficult to treat as they accumulate in the suspended solids of ASP, reducing their bioavailability to microbial degradation (Douben, 2003). Examples of bioaugmentation to enhance removal of PAHs typically focus on the treatment of contaminated soils and groundwater (Vogel, 1996; Straube *et al*., 2003; Yu *et al*., 2005; Jacques *et al*., 2008; Silva *et al*., 2009; Teng *et al*., 2010). Useful knowledge may be gained from these applications, however, since PAHs are mainly associated with the suspended solids in ASPs.

Straube *et al*. (2003) and Sun *et al*. (2014) both considered the role of bioaugmentation and biostimulation for the removal of PAHs from soil (**Table 3**). Biostimulation was applied in order to overcome environmental limitations. Straube *et al*. (2003) demonstrated the ability of the bio-surfactant-producer *Pseudomonas aeraginosa* strain 64 to stimulate the autochthonous PAH degraders in soil samples. After 11 months, bioaugmentation led to an increase in PAH degradation from 23 to 34%. Biostimulation in combination with bioaugmentation however led to an increase in the PAH degradation to 87%. At pilot scale,

after 16 months, PAH removal increased from 12% in the control to 87% with bioaugmentation and biostimulation, although, 86% removal could in fact be achieved with biostimulation alone. Sun *et al*. (2014) found comparable results to Straube *et al*. (2003) when researching the impact of bioaugmentation and biostimulation on former coke works. Over a 3 month period the total PAH levels fell by 24% in the control, 35.9% with bioaugmentation and by 59% with biostimulation. The combination of bioaugmentation and biostimulation only brought about small improvements in comparison to biostimulation alone. The removal of heavy molecular weight PAHs, however, was noticeably higher with bioaugmentation than with biostimulation alone. This is significant due to the increased resistance of heavy molecular weight PAHs to degradation.

6. Discussion and Conclusions

The consistent and stable removal of priority pollutants from industrial wastewater is essential. Whilst close system monitoring and process control are important factors in achieving stable operation and meeting emission limits, operational regimes also need to be economically viable. Even with optimal process control, the inherent variability of industrial wastewaters can still result in emission variability. Compliance with increasingly stringent emission limits therefore requires the application of additional techniques to both meet the required limits and respond to transient treatment issues. Whilst achieving effluents of increasingly high quality is important in the long term, it is equally important that techniques are developed to re-establish treatment promptly after transient events have occurred. Bioaugmentation should be considered as one such technique.

Compliance with nitrogen effluent standards affects a wide variety of industries including palm oil effluent, aquaculture wastewaters, coke making wastewaters and petrochemical wastewaters. Nitrification is well known for its process instability due to the requirement for the close linking of the bacterial species responsible for different parts of the removal process (Philips, Laanbroek and Verstraete, 2002). Low growth rates of nitrifying bacteria and uncoupling of the nitrification chain can be problematic in any treatment, but those of an

503 industrial nature are much more susceptible to disruption as a result of their characteristic
504 variations in loading and the frequent presence of toxic compounds. Bioaugmentation has
505 been shown to offer the potential to stabilise nitrification and in particular to deal with
506 transient treatment problems. Abeysinghe *et al*. (2002) demonstrated the ability of
507 bioaugmentation to improve ammonia removal during stress conditions. Similarly, Ma *et al*.
508 (2009) demonstrated the improved capability of a bioaugmented ASP-treating petrochemical
509 wastewater to deal with shock loadings of COD. Recovery from shock loading was also 50%
510 faster. Compliance can also be problematic for priority pollutants which are persistent and
511 toxic, as the biomass not only requires acclimation but it can still be negatively impacted by a
512 sudden shock load of the toxic compound. As with nitrogen, bioaugmentation has been
513 demonstrated to have some success in the treatment of such compounds. Qu *et al*. (2011)
514 observed improved long-term stability of treatment systems for treating aromatic compounds.
515 The addition of *Pseudomonas* sp. JY-2 led to 90% removal efficiencies compared to 80%
516 without bioaugmentation, with the additional benefit of decreased start-up times. Both Duque
517 *et al*. (2011) and Fang *et al*. (2013) also observed an improved resistance of treatment
518 systems to fluctuating phenol levels with the application of bioaugmentation.

519

520 Despite the benefits which have already been reported, caution must be applied to the
521 findings of the numerous reported investigations. For instance, under the stress conditions
522 reported by Abeysinghe *et al*. (2002), daily dosing was required to maintain sufficient levels
523 of the microorganisms. Bioaugmentation was therefore capable of dealing with transient
524 issues, but would be uneconomic for the long-term maintenance of an unstable treatment
525 system. Similarly, although Ma *et al*. (2009) demonstrated improved nitrogen removal
526 efficiencies, bioaugmentation was conducted in a system with immobilisation and then
527 compared against a conventional reactor. The reduced washout, which was the main benefit
528 of the former system, could therefore potentially have been achieved through the use of
529 carrier media alone, simply supporting biofilm formation. It is important therefore that the
530 purpose of bioaugmentation is clearly defined before success is determined e.g. whether a
531 short-term solution technique or long-term benefits are desired.

532

A significant benefit of bioaugmentation is its ability to treat on demand. Direct dosing can provide an immediate solution to a wide array of failing treatment systems. Where space is an issue and treatment systems are already operating at their maximum capabilities, bioaugmentation may be the only way by which to maintain effluent compliance without resorting to the halting of upstream operations. Direct dosing may make use of commercial products, but these have been associated with a tendency to fail to produce the reported benefits of the product and/or require higher dosing rates than suggested by the manufacturer (Stephenson and Stephenson, 1992). These products may be able to offer a short-term solution to an immediate problem, but because of the problems associated with inadequate adaption of the microorganisms to the environment and the high dosing levels required, they may not be able to meet the requirements for long-term use. As the economic costs associated with treatment processes become more pertinent, the use of 'one-off' dosing may become less viable. The use of side-stream technologies is becoming increasingly common due to their advantages in terms of bacterial adaptation and use in long-term bioaugmentation applications (Krhutková *et al*., 2006; Smith *et al*., 2008; Yu, Peng and Pan, 2012).

Despite some positive reports of the impact of bioaugmentation on process performance, there are still substantial areas that require further research. Firstly, one of the most important aspects requiring research involves the development of an increased understanding of degradation pathways, in the absence of which the possibility of finding a suitable species to inoculate a given compound is reduced. The area of strain development has previously been highlighted for its importance (Thompson *et al*., 2005). It is not only important to consider which strain(s) may be required, but also the requirements of that the strain to operate successfully. Under some circumstances the use of biostimulation may be necessary in order to provide nutrients, or other critical components such as biosurfactants, for the decontamination process to be successful. The synergistic action of a consortium was highlighted by Khehra *et al*. (2005) whilst the importance of the combined action of a biosurfactant and a pre-adapted consortium was reported by Nikolopoulou *et al*. (2013). More research in this field may support the degradation of wastewaters containing polycyclic aromatic hydrocarbons, where complex compounds of different molecular weights are present simultaneously. Developments in genetics may also assist in the development of

564 strains suitable to target xenobiotic compounds for which removal is currently limited;
565 however, concerns around the release of genetically modified bacteria have significantly
566 impacted progress in this area (Davison, 2005). Van Der Gast *et al*. (2003) also reported that
567 treatment performance was more reproducible for a constructed consortium than an
568 undefined community.

569

570 The success of bioaugmentation is increasingly being linked to the effective incorporation of
571 the inoculated strain into the host environment, the success of which is influenced by issues
572 ranging from strain selection and the introduction strategy through to the ability of the strain
573 to survive within the environment to which it is introduced (Herrero and Stuckey, 2014;
574 Thompson et al., 2005). The importance of having a detailed knowledge of the treatment
575 system has been emphasised through numerous applications (Goldstein, Mallory and
576 Alexander, 1985; Bouchez *et al*., 2000; Songzhe *et al*., 2009; Martín-Hernández, Suárez-
577 Ojeda and Carrera, 2012). An understanding of the conditions in a treatment process offers a
578 way in which to prevent an inoculum being negatively influenced by environmental factors
579 such as pH and temperature, as well as exposure to toxic compounds, allowing for the
580 selection of a dosing strategy or location for introduction of the strain to minimise its
581 exposure to negative conditions. Such detailed knowledge can also help inform possible
582 solutions to any problems that may arise. Industries such as dairy processing, where each site
583 encompasses different process operations, would particularly benefit from this approach. As
584 bioaugmentation methodologies can vary greatly, the technique allows for the individuality of
585 different treatment processes to be recognised and catered for.

586

587 Appropriate dosing rates also lack sufficient research. Although many references have been
588 made to over-dosing and/or under-dosing, huge variations can be seen in dose rates that have
589 been successful between applications which appear to be very similar. In the treatment of
590 pyridine and quinoline in laboratory-scale SBRs, both treating wastewater from the same site
591 and achieving a 99% removal rate, Bai *et al*. (2010) reported a dose rate of 0.007–0.0200 g/L
592 in comparison to a dose of 0.223 g/L for Zhang *et al*. (2014b). Of the three species used in
593 each study, two of the species applied were the same in both applications. Whether the

relatively large difference in dose rate can be accounted for by the third species is unknown. Research is also contradictory in the need for repeated inoculations through maintenance dose rates. Both Boon *et al*. (2003) and Abeysinghe *et al*. (2002) reported the need for repeated inoculations via maintenance doses whilst Martín-Hernández, Suárez-Ojeda and Carrera (2012) reported that this was unnecessary if the initial dose rate was sufficiently high to overcome initial survival pressures. High dosing rates have equally been criticised as they have been linked to disturbances in the balance of an ecosystem (Bouchez *et al*., 2000). For this reason, it is important that investigations take place which consider a variety of different dosing regimens for identical wastewater treatment facilities.

The complexity of industrial wastewaters increases the challenge of identifying the most effective techniques, as many interacting processes can take place simultaneously. Despite this, industries should take the opportunity to learn from previous bioaugmentation successes and failures in order to gain from the benefits that may be obtained from bioaugmentation. Research has already increased our understanding of the complex interactions between the introduced microorganisms and the host environment, leading to improved application success. Many of the problems that have arisen in the field of bioaugmentation have been overcome through process development (Error! Reference source not found. **2**).

For the field of bioaugmentation to move forward, it is now essential for key gaps in the research to be addressed. Overall, when considering whether bioaugmentation is successful, the aim of the bioaugmentation process must first be considered i.e. short-term solution to a treatment issue or the long-term improvement of a system. Current research has been limited by the focus on laboratory-scale investigations, synthetic wastewaters and the failure to have adequate controls in place. Understanding in the field would be enhanced significantly by operating parallel studies with control and test process streams. Full-scale investigations have been limited in extent and such investigations have also lacked controls (Parker and Wanner, 2007).

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625

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