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

1. <u>Introduction</u>

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).

54 **Table 1: Industrial Emission Directive emission limits for wastewaters for which an**

55 **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

56

57

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

69

70 **2. Strain selection**

71

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

86

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,

117   2013). Microorganisms can be genetically engineered to over-express degradation genes or to

118   exhibit increased survivability (McClure, Fry and Weightman, 1991; Nüβlein *et al*., 1992;

119   Stroo, Leeson and Herb Ward, 2013). Such techniques enable the possibility of designing

120   microorganisms to assist with the treatment of pollutants which require numerous

121   degradation steps or those required to degrade xenobiotic compounds. Knowledge of the

122   degradation pathways involved for such compounds is limited and a naturally occurring

123   species capable of such degradation may not exist (Stroo, Leeson and Herb Ward, 2013).

124   Microorganisms which have been genetically modified have been investigated in

125   groundwater aquifer microcosms (Jain *et al*., 1987), lake waters (Awong, Bitton and

126   Chaudhryt, 1990) and ASP (McClure, Weightman and Fry, 1989; McClure, Fry and

127   Weightman, 1991). McClure, Weightman and Fry (1989) demonstrated that genetically

128   engineered bacteria were able to survive within a laboratory-scale ASP and did not encourage

129   protozoa reproduction despite large numbers of bacteria being inoculated. Additionally

130   Nüβlein *et al*. (1992) were able to confirm that microorganisms that were genetically

131   engineered were not only capable of surviving in an ASP but were also able to maintain their

132   genetic information and degrade the required pollutants. Such genetic adaptation may allow

133   for the design of microorganisms which are able to assist in the degradation of pollutants

134   which require numerous degradation steps. Further to the genetic modification of

135   microorganisms, gene bioaugmentation, which involves the use of catabolic mobile genetic

136   elements (MGEs), has been highlighted in regard to its applicability to bioaugmentation

137   (Stroo, Leeson and Herb Ward, 2013). Mobile genetic elements consist of pieces of

138   deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) which can be transferred from one

139   organism to another (Stroo, Leeson and Herb Ward, 2013).

140

141   Despite the numerous possible ways in which genetic engineering may improve the future of

142   bioaugmentation, current research is heavily laboratory based and success in the field cannot

143   currently be fully assessed due to legislative restrictions resulting from concerns surrounding

144   the risks to both the environment and human health of the uncontrolled spread of

145   microorganisms which have been genetically engineered (Van Limbergen, Top and

146   Verstraete, 1998). Strategies such as the use of a 'suicide element' and immobilisation

147   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

177 system. Treatment efficiencies and pollutant concentrations, on the other hand, will inform

178 decisions relating to dosing rates (Stephenson and Stephenson, 1992; Park *et al*., 2008).

179

180 **3.1 Dosing technique**

181

182 Direct dosing involves the addition of the selected microorganisms straight into a treatment

183 vessel. Such a technique represents the simplest method of bioaugmentation and can be

184 advantageous in the sense that it can be applied as and when required. This can also be

185 economically beneficial as it does not require plant modification and the associated

186 operational costs. Problematic to this approach, however, is the reduced survival rates of the

187 inoculated microorganisms due to a lack of acclimatisation to the environmental conditions of

188 the host environment resulting, for example, from toxicity, pH, carbon availability, predation

189 and competition between the indigenous and inoculated bacteria (Chong, Pai and Chen, 1997;

190 Bouchez *et al*., 2000; Songzhe *et al*., 2009). The use of a side-stream reactor can help to

191 overcome some of the aforementioned problems as it enables the acclimatisation of the

192 inoculated microorganisms to the environmental conditions, thus increasing their survival

193 rate in the treatment process (Parker and Wanner, 2007). The footprint of a side-stream is

194 typically approximately 10% that of the main reactor. As the side-stream can enable process

195 intensification, this can represent a much smaller investment cost than the cost associated

196 with expanding a treatment works to cope with a higher capacity. Despite this, in some

197 instances the additional land requirements may still be problematic (Salem *et al*., 2003). The

198 use of encapsulation techniques can assist in the incorporation of inoculated bacteria into the

199 existing flocs (Stormo and Crawford, 1992). Bouchez *et al*. (2009) mixed the inoculum with

200 an alginate solution, forming bead structures which were employed in the reactor. The beads

201 allowed the inoculated bacteria to remain in the system and protected them from the intense

202 grazing that was experienced without such encapsulation. The beads were observed to break

203 into fragments by day 8, but such fragments were incorporated into flocs of the indigenous

204 sludge, allowing their successful incorporation into the system. Another recent strategy that

205 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

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

238

239 **3.3 Dosing size and regime**

240

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

256

257 **4. Bioaugmentation failures and associated improvement techniques**

258

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

265   Alexander, 1985; Bouchez *et al*., 2000; Tyagi, da Fonseca and de Carvalho, 2011; Martín-
266   Hernández, Suárez-Ojeda and Carrera, 2012), predation by protozoa (Goldstein, Mallory and
267   Alexander, 1985; Boon *et al*., 2000; Bouchez *et al*., 2000), competition between the
268   inoculated and indigenous bacteria (Stephenson and Stephenson, 1992; Bouchez *et al*., 2000;
269   More *et al*., 2010), the presence of other inhibiting substances (Goldstein, Mallory and
270   Alexander, 1985; Bouchez *et al*., 2000; Tyagi, da Fonseca and de Carvalho, 2011), the
271   availability of alternative substrates (Goldstein, Mallory and Alexander, 1985; Chitra *et al*.,
272   1995; Quan, Shi, Liu, Wang, *et al*., 2004; Mahin *et al*., 2011), the need for an acclimatisation
273   period (Stephenson and Stephenson, 1992) and extremes in environmental factors such as
274   temperature and pH (Tyagi, da Fonseca and de Carvalho, 2011). An understanding of the root
275   cause of the failure of the bioaugmentation process is important to ensure the advancement of
276   bioaugmentation applications.

277

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

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

298

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

307

308 Nutrient limitation is a particularly important factor in the treatment of industrial wastewaters
309 which frequently lack the essential nutrients required for microbial development (Burgess,
310 Quarmby and Stephenson, 1999). Nutrient limitations have been held responsible for failed
311 bioaugmentation attempts due to the competition between the indigenous and inoculated
312 bacteria. Ramadan, El-Tayeb and Alexander (1990) demonstrated that the supplementation of
313 nutrients could increase the likelihood of a successful bioaugmentation outcome as the
314 addition of nitrogen and phosphate allowed for low densities of inoculum to remove p-
315 nitrophenol (PNP), potentially increasing the inoculum growth rates and resistance to higher
316 protozoa numbers. Such nutrient addition is referred to as biostimulation. Biostimulation,
317 however, can also include the addition of other stimulants such as surfactants. Nikolopoulou,
318 Pasadakis and Kalogerakis (2013) demonstrated that the presence of a biosurfactant could
319 increase degradation rates in oil-contaminated sites by enhancing the solubility of the
320 hydrocarbons. Under such treatment systems it is important, however, to have adequate
321 controls in place in order to be able to assess to what degree the improvement is a result of
322 the biostimulation rather than a result of the bioaugmentation itself. Due to its complementary
323 action, biostimulation has therefore become a technique that is frequently reported for use
324 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

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

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

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

Table 3: Examples of bioaugmentation applied to compounds present in industrial
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

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

446

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

456

457 Polycyclic aromatic hydrocarbons (PAHs) can be found in oil and gas wastewaters as well as
458 coke-making wastewaters and are typically difficult to treat as they accumulate in the
459 suspended solids of ASP, reducing their bioavailability to microbial degradation (Douben,
460 2003). Examples of bioaugmentation to enhance removal of PAHs typically focus on the
461 treatment of contaminated soils and groundwater (Vogel, 1996; Straube *et al.*, 2003; Yu *et*
462 *al.*, 2005; Jacques *et al.*, 2008; Silva *et al.*, 2009; Teng *et al.*, 2010). Useful knowledge may
463 be gained from these applications, however, since PAHs are mainly associated with the
464 suspended solids in ASPs.

465

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

473   after 16 months, PAH removal increased from 12% in the control to 87% with

474   bioaugmentation and biostimulation, although, 86% removal could in fact be achieved with

475   biostimulation alone. Sun *et al*. (2014) found comparable results to Straube *et al*. (2003)

476   when researching the impact of bioaugmentation and biostimulation on former coke works.

477   Over a 3 month period the total PAH levels fell by 24% in the control, 35.9% with

478   bioaugmentation and by 59% with biostimulation. The combination of bioaugmentation and

479   biostimulation only brought about small improvements in comparison to biostimulation

480   alone. The removal of heavy molecular weight PAHs, however, was noticeably higher with

481   bioaugmentation than with biostimulation alone. This is significant due to the increased

482   resistance of heavy molecular weight PAHs to degradation.

483

484   **6. Discussion and Conclusions**

485

486   The consistent and stable removal of priority pollutants from industrial wastewater is

487   essential. Whilst close system monitoring and process control are important factors in

488   achieving stable operation and meeting emission limits, operational regimes also need to be

489   economically viable. Even with optimal process control, the inherent variability of industrial

490   wastewaters can still result in emission variability. Compliance with increasingly stringent

491   emission limits therefore requires the application of additional techniques to both meet the

492   required limits and respond to transient treatment issues. Whilst achieving effluents of

493   increasingly high quality is important in the long term, it is equally important that techniques

494   are developed to re-establish treatment promptly after transient events have occurred.

495   Bioaugmentation should be considered as one such technique.

496

497   Compliance with nitrogen effluent standards affects a wide variety of industries including

498   palm oil effluent, aquaculture wastewaters, coke making wastewaters and petrochemical

499   wastewaters. Nitrification is well known for its process instability due to the requirement for

500   the close linking of the bacterial species responsible for different parts of the removal process

501   (Philips, Laanbroek and Verstraete, 2002). Low growth rates of nitrifying bacteria and

502   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

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

548

549   Despite some positive reports of the impact of bioaugmentation on process performance,
550   there are still substantial areas that require further research. Firstly, one of the most important
551   aspects requiring research involves the development of an increased understanding of
552   degradation pathways, in the absence of which the possibility of finding a suitable species to
553   inoculate a given compound is reduced. The area of strain development has previously been
554   highlighted for its importance (Thompson *et al*., 2005). It is not only important to consider
555   which strain(s) may be required, but also the requirements of that the strain to operate
556   successfully. Under some circumstances the use of biostimulation may be necessary in order
557   to provide nutrients, or other critical components such as biosurfactants, for the
558   decontamination process to be successful. The synergistic action of a consortium was
559   highlighted by Khehra *et al*. (2005) whilst the importance of the combined action of a
560   biosurfactant and a pre-adapted consortium was reported by Nikolopoulou *et al*. (2013). More
561   research in this field may support the degradation of wastewaters containing polycyclic
562   aromatic hydrocarbons, where complex compounds of different molecular weights are
563   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

594 relatively large difference in dose rate can be accounted for by the third species is unknown.
595 Research is also contradictory in the need for repeated inoculations through maintenance dose
596 rates. Both Boon *et al*. (2003) and Abeysinghe *et al*. (2002) reported the need for repeated
597 inoculations via maintenance doses whilst Martín-Hernández, Suárez-Ojeda and Carrera
598 (2012) reported that this was unnecessary if the initial dose rate was sufficiently high to
599 overcome initial survival pressures. High dosing rates have equally been criticised as they
600 have been linked to disturbances in the balance of an ecosystem (Bouchez *et al*., 2000). For
601 this reason, it is important that investigations take place which consider a variety of different
602 dosing regimens for identical wastewater treatment facilities.

603 The complexity of industrial wastewaters increases the challenge of identifying the most
604 effective techniques, as many interacting processes can take place simultaneously. Despite
605 this, industries should take the opportunity to learn from previous bioaugmentation successes
606 and failures in order to gain from the benefits that may be obtained from bioaugmentation.
607 Research has already increased our understanding of the complex interactions between the
608 introduced microorganisms and the host environment, leading to improved application
609 success. Many of the problems that have arisen in the field of bioaugmentation have been
610 overcome through process development (Error! Reference source not found. **2**).
611

612 For the field of bioaugmentation to move forward, it is now essential for key gaps in the
613 research to be addressed. Overall, when considering whether bioaugmentation is successful,
614 the aim of the bioaugmentation process must first be considered i.e. short-term solution to a
615 treatment issue or the long-term improvement of a system. Current research has been limited
616 by the focus on laboratory-scale investigations, synthetic wastewaters and the failure to have
617 adequate controls in place. Understanding in the field would be enhanced significantly by
618 operating parallel studies with control and test process streams. Full-scale investigations have
619 been limited in extent and such investigations have also lacked controls (Parker and Wanner,
620 2007).

621

622

625

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