1 **Induction of microbial oxidative stress as a new strategy to enhance the enzymatic**

2 **degradation of organic micropollutants in wastewater**

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16 Organic micropollutants (OMPs) are pervasive anthropogenic contaminants of fresh and

17 marine waters with known potential to adversely affect aquatic life (e.g. endocrine disruption).

18 Their ubiquitous environmental occurrence is primarily attributed to wastewater treatment

19 plant discharges following their incomplete removal by common biological treatment

20 processes. This study assesses a new strategy for promoting the degradation of six model OMPs

21 (i.e. sulfamethoxazole, carbamazepine, tylosin, atrazine, naproxen and ibuprofen) by

22 stimulating microbial oxidoreductase production to counter the effects of oxidative stress

23 caused by oxygen perturbation. Microbial cultures from dairy farm wastewater were exposed

24 to a cyclical ON-OFF perturbations of oxygen supply, ranging from 0.16 to 2 cycles per hour

25 (i.e. 2, 1, 0.5, 0.25 and 0.16 cycles/hour), in laboratory bioreactors. The activity and relative

26 abundances of microbial oxidoreductases (such as peroxidases, cytochromes P450) were

27 upregulated by oxygen perturbation. In comparison to controls subjected to constant oxygen

28 levels, OMP concentrations in perturbed cultures decreased by $70\pm9\%$ (mean $\pm$ SD). A

29 distance-based linear model confirmed strong positive correlations between the relative

30 abundance of the bacterial families, *Rhodocyclaceae, Syntrophaceae* and

31 *Syntrophobacteraceae,* and oxygen perturbations. Our results confirm that intentional

32 perturbation of oxygen supply to induce microbial oxidative stress can improve OMP removal

33 efficiencies in wastewater treatment bioreactors.

34 Microbial communities ∣ Oxidoreductase activity ∣ Bioreactor aeration regime ∣ Wastewater

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Significance

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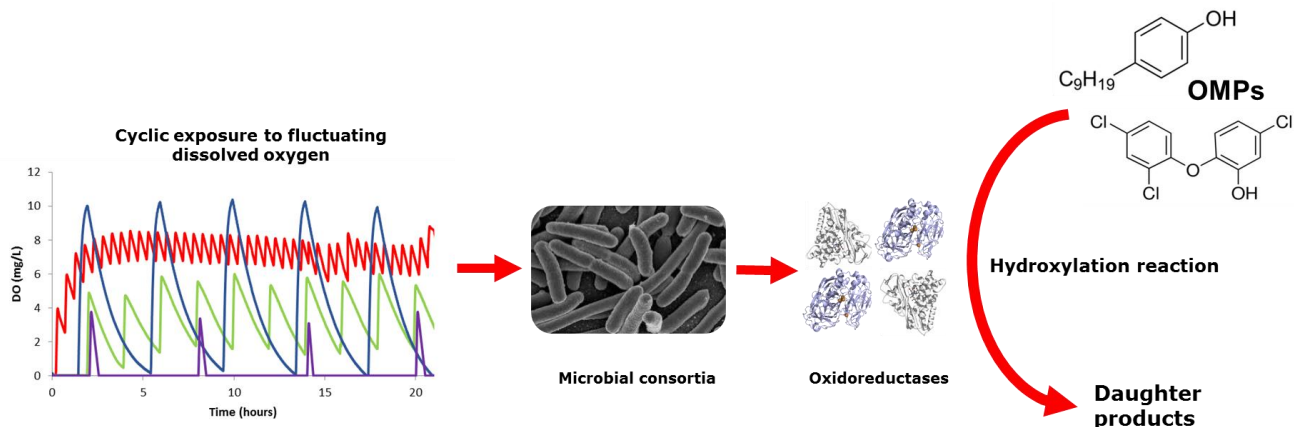
48 Oxygen concentration is a critical control variable for biological processes in conventional

49 wastewater treatment systems. We provide a simple approach that could be used in existing

50 wastewater treatment infrastructure to enhance removals of otherwise persistent OMPs by

51 exploiting oxidative stress responses of microbes. Our study illustrates the ability of fluctuating

52 oxygen conditions to affect microbial activity and polulation by upregulating oxidoreductases

53 linked to the removal of organic micropollutants. The incorporation of inexpensive and easy to

54 implement dissolved oxygen supply strategies for more efficient OMPs biotransformation

55 provides a realistic approach to improving the treatment performance of biological processes.

56

Introduction

58 The discharge of trace levels of pharmaceuticals, industrial chemicals, pesticides and personal

59 care products into aquatic environments is an issue of global environmental concern (1–3).

60 Because of their resistance to conventional wastewater treatment and environmental

61 persistence as complex mixtures, these organic micropollutants (OMPs) present risks of

62 adverse ecological impact to biota via direct toxicity and endocrine disruption (4–6). For

63 example, occurrence of the herbicide, atrazine, even at concentrations below 6 µg/L in

64 receiving waters, caused behavioural changes in the zebrafish, *Brachydanio rerio,* such as a

65 preference for dark habitats and alterations in swimming due to failure of its nervous system

66 (7). OMP exposure effects may be additive; for example studies by (8) and (9) have reported

67 that exposure of female *Danio rerio* to pharmaceutical mixtures of acetaminophen,

68 carbamazepine, gemfibrozil and venlafaxine significantly reduces fecundity, and

69 disproportionate to effects expected for individual exposures. Such concerns about the

70 environmental occurrence of OMPs have triggered attempts to enhance their biodegradation

71 during wastewater treatment (10). Although conventional activated sludge methodologies are

72 not specifically optimised to eliminate OMPs (11), exploiting the diverse enzymatic potential

73 of microbial consortia has been identified as a critical pathway for OMP removal (12–14).

74

75 In the period since the first oxidative conditions arose on Earth due to early photosynthesis,

76 microbes have evolved to survive exposure to a variety of harmful oxidative stresses (15). For

77 instance, oxidative stress generated by the high concentration of intracellular reactive oxygen

78 species (ROS) including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals

79 ($OH^.$), and *ipso facto*, induces oxidative enzyme gene expression (16) and consequent synthesis

80 of antioxidative enzymes such as peroxidases and oxidoreductases to protect against such

81 oxidative stress (17, 18). From a wastewater treatment perspective, intentional manipulations

82 of environmental oxygen concentrations could stimulate the production of these enzymes,

83 some of which are also capable of degrading toxic substances including polycyclic aromatic

84 hydrocarbons and organophosphorus contaminants (19–23). Therefore, oxygen perturbations

85 might pre-dispose cells, through transcriptional responses of their oxidoreductases to protect

86 against iterative oxidative stress, to enhance OMP degradation. This proposed mechanism

87 implies that simple oxygen control strategies within wastewater treatment bioreactors may

88 provide a cost-effective solution for enhanced biological OMP removal.

89 In this study we investigated the impact of dynamic variation in oxygen concentration on OMP

90 biotransformation by representative wastewater microbial consortia. We hypothesise that

91 varying dissolved oxygen concentration can enhance the degradation of OMPs by inducing

92 oxidative stress and forcing microbes to produce oxidoreductases. Specifically, we assessed

93 the effect of varying frequency of oxygen perturbations on microbial consortia by monitoring

94 the synthesis of oxidative biocatalysts (oxidoreductases) along with the removal of OMPs.

95

Results

Influence of oxygen perturbations on microbial enzymatic activity

Five frequencies (2, 1, 0.5, 0.25 and 0.16 cycles per hour) were tested at two aeration regimes (high-DO, up to 10 mg- DO /L and low-DO, up to 4 mg- DO /L) that generated cyclic patterns of dissolved oxygen concentrations in bioreactors (Figure 1, Table 1). The oxidoreductase (lignin peroxidase, horseradish peroxidase, laccase and cytochrome P450) and hydrolase (beta-glucosaminidase, beta-glucosidase) activities in bioreactors were detected spectrophotometrically by the oxidation of their respective chromogenic substrates (Methylene Blue [MB], Azure B [AB], 3,4-Dihydroxy-L-phenylalanine [L-DOPA], 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)[ABTS], Sudan Orange G [SO], 4-nitrophenyl-dodecanoate [pNP-12], Indole and 4-Aminoantipyrine [4-AAP], 4-nitrophenyl N-acetyl-β-D-glucosaminide [pNP-A] and 4-nitrophenyl- β-D-glucopyranoside [pNP-G] (Table S1). Cultures perturbed in high-DO regimes at 0.25 cycles/hr showed significantly higher (ANOVA, $p < 0.05$) beta-glucosidase (pNP-G), lignin peroxidase (AB, MB) and horseradish peroxidase (ABTS) activity compared to cultures treated at 1 (lignin peroxidase (AB, MB), beta-glucosidase (pNP-G) and cytochrome P450 (Indole)) or 2 (lignin peroxidase (AB, MB) only) cycles/hr. The cultures exposed to 0.25 and 0.5 cycles/hr and maintained within a low-DO range showed high (ANOVA, $p < 0.05$) cytochrome P450 (Indole, 4-AAP), lignin peroxidase (AB, MB), and horseradish peroxidase (L-DOPA, ABTS) activity whereas 0.16 cycles/hr subjected cultures exhibited more beta-glucosaminidase (pNP-A), beta-glucosidase (pNP-G), lignin peroxidase (MB, AB) and horseradish peroxidase (L-DOPA, ABTS) activity (Figure 2a).

By contrast, under constant non-perturbed oxygen concentrations, the high-DO aerobic cultures exhibited greater (ANOVA, $p < 0.05$) cytochrome P450 (pNP-12), beta-glucosaminidase (pNP-A), beta-glucosidase (pNP-G), lignin peroxidase (AB) and horseradish peroxidase (L-DOPA) activity, while the low-DO aerobic samples showed greater laccase (SO), beta-glucosaminidase (pNP-A), lignin peroxidase (MB) and horseradish peroxidase (ABTS, L-DOPA) activity. Proteomic analysis supported the above enzyme activity profiles with significant (95% confidence interval) observations showing that maximum relative abundance of expressed oxidoreductases (cytochromes, peroxidases and oxidases) had occurred in perturbed low-DO cultures (0.5 and 0.25 cycles/hr) (Figure 2b).

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Micropollutant removal by mixed cultures

129

130 The removal of OMPs (at 0.1 mg/L initial nominal concentrations) was significantly (ANOVA,

131 $p < 0.05$) increased by the perturbed DO conditions (removing OMPs below the limit of

132 quantification (LOQ)). The perturbed treatments showed 70±9% (mean ± SD) removal of

133 sulfamethoxazole, carbamazepine, tylosin, atrazine, naproxen and ibuprofen, with an average

134 residual concentration of 0.04 mg/L for individual OMPs remaining at the end of the

135 experiments. Cultures exposed to 0.5 cycles/hr in the low-DO regime exhibited near log-

136 removal (~0.01-0.02 mg/L residual concentrations) of sulfamethoxazole, naproxen and

137 ibuprofen, whereas carbamazepine, tylosin and atrazine concentrations in the cultures

138 subjected to 0.16 and 0.25 cycles/hr in low-DO range were attenuated to residuals of 0.012-

139 0.04 mg/L (Figure 3). Cultures under constant low-DO conditions showed 35±8% removal of

140 all compounds compared to constant high-DO treated cultures with 25±6% removal. On

141 average, 0.06 mg/L residual concentrations of OMPs were detected in constant low-DO

142 cultures while constant high-DO cultures exhibited 0.08 mg/L residual concentration (Figure

143 3). There were no significant differences (post-hoc Tukey test, $p > 0.05$) for sulfamethoxazole,

144 carbamazepine and tylosin between DO perturbed and non-perturbed constant high and low-

145 DO aerobic cultures. However, a significant removal (post-hoc Tukey test, $p < 0.05$) of

146 atrazine, naproxen and ibuprofen can be seen under low-DO aerobic perturbed cultures

147 compared to high-DO aerobic and non-perturbed constant cultures. Autoclaved biomass, used

148 as a negative control, showed a loss of 15±6% for all OMPs (data not shown) which is attributed

149 to abiotic factors such as hydrophobic partitioning and sorption to the sludge solids. Figure S1

150 and Tables S2 and S3 in the supplementary information show OMP removal, OMP residual

151 concentrations and quality control measures, respectively.

152

153 **Influence of oxidative stress on microbial speciation**

154 A total of 527 operational taxonomic units (97% OTUs) were identified in the cultures, of

155 which 513 represented Bacteria and 13 were Archaea. The percentage occurrence of bacteria

156 belonging to the phylum Proteobacteria, which are linked to the biotransformation of

157 recalcitrant contaminants in wastewaters (24), predominates and correlated with respect to

158 ecological variables like process operations (changing oxygen conditions), influent wastewater

159 characteristics or geographical location (25). *Comamonadaceae* (40%) was the dominant

160 family in cultures perturbed with 0.25 cycles/hr in high-DO regime while a higher abundance

161 of *Methylophilaceae* (25%) was found in 1 and 2 cycles/hr treated cultures at high-DO range

162 (Figure S2 in the supplementary information). Cultures exposed to 0.16 cycles/hr in low-DO

163   regime showed a higher abundance of *Rhodocyclaceae* (15%). *Flavobacteriaceae* (10-12%)

164   and *Pseudomonadaceae* (14%) were dominant in cultures perturbed with 0.25 and 0.5 cycles/hr

165   at low-DO regime. The non-perturbed constant high and low-DO regime subjected cultures

166   resulted in higher abundances (30-45%) for *Comamonadaceae*. dbRDA constrained ordination

167   based on Bray-Curtis similarity was conducted to determine the extent to which bacterial

168   community variation at family level can be explained by DO conditions (Figure 4). A linear

169   relationship was confirmed between dependent variables (bacterial families, enzyme activity

170   and residual OMP concentration) and changes in predictor variable (perturbed and non-

171   perturbed DO conditions). The most important parameters contributing variation in bacterial

172   community composition, assessed at the level of family was the presence of the bacterial

173   families *Rhodocyclaceae, Syntrophaceae*, *Syntrophobacteraceae Sphingobacteriaceae*,

174   *Xanthomonadaceae, Campylobacteriacea* and *Aeromonadaceae*, as well as laccase, lignin,

175   horseradish peroxidase and cytochrome P450 activity, and OMPs (namely sulfamethoxazole,

176   carbamazepine, naproxen and ibuprofen; Figure 4). PERMANOVA indicating the similarities

177   and differences between the different DO treatments in terms of bacterial composition, enzyme

178   activity and OMP removal efficiencies showed significant difference (F = 3.26; df = 10, 11; p

179   = 0.001) between perturbed and non-perturbed cultures. Variation in bacterial community

180   composition positively correlated with microbial enzyme activity (relate analysis, R = 0.46, p

181   < 0.05) and OMP residuals (relate analysis, R = 0.54, p < 0.05).

182

183   **Discussion**

184   **Microbial Community Composition**

185   Biological processes in waste treatment are manipulated by changing operation conditions (26)

186   to promote specific eco-physiological characteristics of the microbial community to achieve

187   the desired outcomes (25). Previously, the occurrence and abundance of Proteobacteria phyla

188   has been linked to fluctuating concentrations of DO (27) and the effective biotransformation

189   of pharmaceuticals (28). In the present study, perturbations of DO conditions have translated

190   to relative differences in the microbial community composition. The core set of bacterial

191   families   included   were   *Commamonadaceae,   Flavobacteriaceae,   Rhodocyclaceae,*

192   *Sphingobacteriaceae,   Xanthomonadaceae,   Burkholderiaceae   and   Pseudomonadaceae*,

193   identified previously as major contributors to activated sludge treatment within wastewater

194   treatment plants (WWTP) worldwide (29, 30). Genomic analysis has revealed that many

195   common wastewater microorganisms have considerable potential to produce specialised

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196 enzymes and metabolites with OMP degradation potential (31–34). The core bacterial families

197 observed in our study from the phylum Proteobacteria have been reported as organic

198 micropollutant degraders in WWTPs treating pharmaceutical, petroleum refinery, domestic

199 and agricultural wastewater (24). *Commamonadaceae* represented ~40% of the bacteria in both

200 perturbed (0.25 cycles/hr, high-DO) and non-perturbed constant high-DO aeration regimes,

201 followed by *Rhodocyclaceae* and *Syntropahceae* (15%) in 0.16 cycles/hr, low-DO perturbed

202 conditions. These families can degrade aromatic compounds and denitrify industrial

203 wastewaters (35). *Syntropahceae* dominated the anaerobic conditions within the 0.16 cycles/hr

204 perturbed DO regime, consistent with other observations (36). However, *Flavobacteriaceae,*

205 *Pseudomonadaceae* and *Sphingomonadaceae* (10-14%) families evident in 0.25 and 0.5

206 cycles/hr perturbed cultures at low-DO regime were identified as the core bacteria in activated

207 sludge systems with floc-formation capabilities (37) and capable of co-metabolic

208 biotransformation of ibuprofen (38), carbamazepine and sulfamethoxazole (39, 40). We found

209 a significant correlation between microbial communities, enzyme activity, and OMP residual

210 concentrations under different DO treated cultures. The bioremediation potential of laccases

211 produced by *Pseudomonadaceae* and *Xanthomonadaceae* was successfully demonstrated (41).

212 The cytochrome P450 system expressed in *Rhodococcus* spp. is known to degrade a range of

213 wastewater-derived steroid hormones and toxic herbicides (42).

214

Enzyme activity

216 Microbial enzymes are promising biocatalysts for OMP degradation (43); however, controlling

217 the synthesis of poorly characterized enzymes to specifically enhance OMP treatment in

218 complex wastewater communities has not been achieved. Oxygen is an obvious candidate for

219 directed biocatalyst synthesis because it has a pronounced impact on the physiology and

220 metabolism of microorganisms, including the induction of oxidoreductases demonstrated to

221 degrade OMPs in pure enzyme assays. In this study, we found that cycling between aerobic

222 and anaerobic phases stimulated the constant production of oxidoreductases (peroxidases,

223 cytochromes and laccases) and native activated sludge enzymes such as hydrolases (beta-

224 glucosidase and beta-glucosaminidase). The bacterial response to changing environmental

225 oxygen was previously demonstrated by the activation of global regulatory systems – Arc BA,

226 Fnr, OxyR and Sox RS (Figure 5) (44). The expression of peroxidases and cytochrome P450

227 in our study was approximately 1-2 orders of magnitude higher in the cultures perturbed with

228 0.25 and 0.5 cycles/hr at low-DO regime compared to 0.25 and 1 or 2 cycles/hr at high-DO

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229 regime treated cultures. Thus, exposing microbes to fluctuating aerobic-anaerobic phases
230 induces the cellular enzymatic machinery to activate genetic adaptive strategies (e.g. *SoxRS*
231 and *OxyR* mediated oxidative stress response systems). Gene activation leads to the synthesis
232 of scavenging oxidoreductases, which provide a feedback control to lower the intracellular
233 ROS concentrations (16, 45) (Figure 5). Increasing oxygen concentrations seem to result in the
234 accumulation of significant amounts of intracellular ROS which overwhelm the cell with
235 additional peroxidase expression. Increased levels of these enzymes are unable to completely
236 counterbalance the toxic action of free oxygen radicals (46). However, with decreasing oxygen
237 concentrations, anti-oxidative genes may remain induced and produce more active and efficient
238 oxidoreductases (peroxidases and cytochromes) (47). The synthesis of beta-glucosidase is
239 consistent with their involvement in organic matter degradation (48). Switching between
240 aerobic and anaerobic conditions also yields bacterial metabolic reprogramming by regulating
241 the activation of one component Fnr oxygen sensor and the two-component Arc system at the
242 transcriptional level (44). The Fnr and Arc A systems exhibit maximal activity under
243 microaerobic and anaerobic conditions (oxygen concentration $\leq 2.5\%$ of total gaseous volume)
244 leading to the mediation of metabolic responses, amplified production of redox signals, and
245 more endogenous ROS generation (44, 49–51) (Figure 5). The regulation and expression of the
246 oxidative genes by these regulatory systems allows a sensitive but robust transcriptional
247 response over a wide range of oxygen concentrations. Proteomic analysis supported the
248 correlation between maximum synthesis of cytochromes and peroxidases in the perturbed low-
249 DO regime cultures. The synthesised oxido-reductases can simultaneously consume ROS as a
250 cellular defence mechanism while oxidizing OMPs (52, 53). More studies would be needed to
251 determine how ROS at cellular concentrations activates the expression of anti-oxidative genes
252 and synthesis of oxidoreductases linked to the catalysis of OMPs.

Organic micropollutant removal

254 The efficiency of OMP biotransformation is linked to environmental conditions that enhance
255 the expression of oxidoreductases by microbes (54). Such outcomes are consistent with results
256 of this study where enhanced oxidoreductase production and OMP removal was observed in
257 DO perturbed cultures compared to non-perturbed controls and autoclaved biomass. Similarly,
258 sequential induction of aerobic and anaerobic conditions during biological wastewater
259 treatment can enhance OMP biotransformation (55). Sludge cultures exposed to perturbations
260 (0.16, 0.25, 0.5, 1 and 2 cycles/hr) in both low and high-DO regime showed more OMPs
261 removal compared to controls with constant oxygen supply (Figure 3).

262   These data indicate that synthesis of highly active and efficient oxidoreductases (cytochrome
263   P450, lignin and horseradish peroxidase) and hydrolases (beta-glucosidase) as a result of
264   induced oxidative stress is a mechanism to exploit for OMP attenuation (56). Changing the
265   oxygen conditions can influence the redox potential of the cell, hence, resulting in endogenous
266   ROS production. As previously described, the oxygen free radicals can act as co-substrates for
267   the synthesis of oxidoreductases (peroxidases, laccases and cytochromes) while fortuitously
268   oxidising nearby organic substrates (53, 56). For example, the synthesised lignin peroxidases
269   are high redox peroxidases which use activated molecular oxygen (such as superoxides and
270   hydrogen peroxides) for the hydroxylation and cleavage of aromatic rings (57–59). We suggest
271   that non-specific oxidoreductases such as peroxidases, cytochrome P450 and laccases in the
272   DO perturbed cultures are metabolising OMPs in the presence of energy molecules (NADH or
273   NAD$^+$) produced during cellular metabolism operated by different oxygen regulatory systems
274   (53).

275   The capability of synthesised oxidoreductases for OMP biotransformation across different
276   functional groups including carboxylic compounds (NPX and IBP), hydroxyl compounds (TT)
277   and other functional groups (SMX, CBZ and ATZ) is demonstrated by their transformation in
278   the DO perturbed cultures (53, 60, 61). The active haem and copper centres of peroxidases and
279   laccases and their reactivity with the evaluated OMPs (having relatively small sizes being
280   comprised of 1-2 benzene rings) explains their significant biotransformation. The lower OMP
281   removal efficiency of non-perturbed cultures is consistent with the generation of excess
282   endogenous ROS, which partially inactivates peroxidases and allows for spontaneous
283   conversion back into the resting enzyme (59). To improve the biodegradability of otherwise
284   recalcitrant pharmaceuticals, personal care products and other micropollutants, the key step is
285   to induce microorganisms to produce degradative enzymes. The efficient biodegradation of
286   acetaminophen, acetyl sulfamethoxazole, atenolol and naproxen by oxidoreductases has been
287   reported (39, 62), and 48-99% enzymatic transformation of carbamazepine, a highly
288   recalcitrant molecule, by cytochrome P450 was demonstrated (63). The easy implementation,
289   cost-effectiveness and potential to degrade a wide range of OMPs and other pollutants of
290   concern suggests that this mechanism should be further investigated and exploited for its
291   treatment potential.

292

293   This study suggests that exposing wastewater microbial species to oscillating oxygen
294   concentrations in different aeration regimes can affect bacterial species selection, induce *de*

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295 *novo* synthesis of oxidative biocatalysts and subsequently enhance OMP removals. The use of

296 such dissolved oxygen perturbations implies new and underutilized mechanisms for

297 wastewater treatment plants to use existing infrastructure to improve their treatment

298 efficiencies for problematic organic contaminants (14). With a potential to catalyse OMP

299 biotransformations, the use of induced oxidoreductases in wastewater treatment has just started

300 to be investigated at the laboratory scale (64). In future, the biodegradation of OMPs may be

301 achieved by improving the performance of existing wastewater treatment systems under

302 conditions that provide directed environmental stresses that result in over-production of

303 beneficial OMP-degrading biocatalysts.

304

Materials and methods

306 Additional details of the materials and methods can be found in the supplementary information.

Reactor operational parameters and sampling

308 Fed-batch experiments were performed with mixed liquor sludge from an oxidation pond near

309 the stables and milking areas of a dairy farm in Alfriston, New Zealand. Sludge was diluted to

310 3 g/L volatile suspended solids (VSS) with distilled water to a total volume of 1L. The

311 bioreactors were continuously fed with ten times concentrated 100 ml synthetic wastewater

312 (400 mg/L of COD as acetate and 60 mg-N/L ammonium as a nitrogen source), also containing

313 0.1 mg/L each of six model OMPs (detailed in the supplementary information) provided at a

314 constant feed rate of 0.034 mL/min. for 48 hours. Oxidative stress was imposed on cultures by

315 iteratively exposing them to ON-OFF cycles of an oxygen enriched air supply during the

316 cultivation period. Additionally, constant (non-perturbed) high and low-DO conditions and

317 autoclaved (dead) biomass were tested as controls. Each condition was tested in duplicate with

318 sampling at the end of the cycle.

Enzymes activity assays

320 The activity of oxidoreductases in culture biomass samples was determined

321 spectrophotometrically by measuring the degradation (oxidation and hydrolysis) of various

322 chromogenic substrates used as surrogate xenobiotics (Table S1 in the supplementary

323 information). In brief, 20 ml aliquots of microbial culture samples were centrifuged at 18,078

324 x g for 15 min. at -4°C in 50 ml falcon tubes. The pellets from each tube were resuspended in

325 either 10 ml acetate buffer or 10 ml phosphate buffer (detailed in supporting information). Each

326 well of a 96 well microplate was filled with 100 µl aliquots of the respective buffers, 100 µl

327   chromogenic dye and 100 µl resuspended biomass. Dyes and samples resuspended in double

328   the amount of buffer served as controls. The prepared plates were mixed and incubated at 30˚C

329   for 1 hour. To start reactions of the sample enzymes with assay dyes, 10 µl of 0.3% H_2O_2 at

330   30% was added to the Methylene Blue, Azure B, L-DOPA and ABTS dye wells and 10 µl of

331   1M NaOH added to the para-nitrophenol dye wells (to stop the reaction) and vortex mixed.

332   Changes in absorbance caused by chromogenic reactions were read on a Victor X3 Multimode

333   Plate Reader (PerkinElmer, USA) at different wavelengths (refer to supporting information).

334   **Protein extraction and identification**

335   Protein extraction was performed by cell lysis followed by precipitation of proteins and

336   separation of low and high stringency protein fractions by 1D SDS PAGE. Briefly, 30 ml of

337   sludge sample was pelleted and washed with 50 ml of 0.9% sodium chloride and spun down at

338   20,200 xg for 20 min at -4°C. The pellet was then washed again in 40 ml Tris-HCl (pH-7) and

339   centrifuged at 20,200 xg for 20 min at -4°C to get the final pellet, which was resuspended in

340   sample buffer, pulse-vortexed and, then placed on ice for 2 h with regular mixing at 15 min

341   intervals. Samples were sonicated for 15 s (six rounds on ice) and centrifuged at 20,200 xg for

342   3 min at 4°C. The supernatant was mixed with trichloroacetic acid (100% w/v) and centrifuged

343   at 23,400 xg for 30 min at -4°C after which the pellet was washed with 5 ml cold acetone twice.

344   The final pellet was heat dried and re-suspended in 400 µl low stringency buffer (see

345   supplementary information) to generate a low stringency fraction (LSF) in the supernatant,

346   followed by centrifugation, then extracting this pellet in 400 µl high stringency buffer to

347   generate a high stringency fraction (HSF). Proteins in both fractions were quantified

348   spectrophotometrically by fluorescence. Crude separation of LSF and HSF proteins was

349   achieved using 1D SDS PAGE. Individual bands were excised with a sharp razor blade and

350   placed into low-binding, siliconized microcentrifuge tubes for destaining reduction, alkylation

351   and trypsinolysis. Finally, 5 µL of the generated tryptic peptides of the microbial proteins were

352   injected onto a SCIEX 6600 triple TOF mass spectrometer. Protein identification was carried

353   out by comparing the obtained peptide sequences against those of the UniProt database.

354   Additional methods and details can be found in the Supplementary Information.

355

356   **Extraction and analysis of organic micropollutants (OMPs)**

357   OMPs at 0.1 mg each per L were prepared from a 1 g each per L stock solution in methanol

358   and fed continuously at a feed rate of 0.034 mL/min to the bioreactor cultures for 48 h. Residual

359   OMPs were extracted from the cultures using solid phase extraction (SPE) on OASIS HLB

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360   cartridges (Waters, Milford, MA, USA) following the method described by (65). Briefly, 200

361   mL of aqueous sludge culture was collected at the end of the cultivation cycle and centrifuged

362   to remove suspended particles (20,4000 xg for 20 min at -4°C). The supernatant was passed

363   through the cartridges (pre-conditioned with 5 mL each of tert-methyl butyl ether, methanol

364   (Merck; laboratory grade purity) and deionised water) and washed with water. The OMPs were

365   extracted in methanol (10 mL) under a vacuum system.

366   OMP analysis was performed by liquid chromatography coupled with mass spectrometry (LC-

367   MS) using a Shimadzu 2020 Series LC-MS (Shimadzu, Japan) with an Agilent ZORBAX

368   Eclipse Plus C18 column (2.1 mm × 100 mm, particle size 1.8 µm, Agilent Technologies,

369   Germany) following EPA Method 1694 (66). A binary gradient system of mobile phase A,

370   0.1% formic acid in deionised water, and mobile phase B, 0.1% formic acid in acetonitrile were

371   used to separate analytes in positive ESI mode, while 5 mM ammonium acetate, pH-5.5 (mobile

372   phase A) and methanol (mobile phase B) were used for analysis in negative ESI mode. The

373   solvent gradient programme for positive ESI mode was as follows: 10% B to 60% B in 24 min

374   and then maintained at 100% B for 30 min. For negative ESI mode, the gradient started with

375   40% B, and was increased linearly to 100% B over 13.5 min, and then maintained at 40% B

376   for 17 min. The flow rate was 0.2 ml/min. in both modes and the injection volume was set to 2

377   µl and 10 µl for +/- polarity modes, respectively. Limit of detection and limit of quantification

378   were determined using signal/noise ratios of 3 and 10 respectively.

379   **Microbial DNA isolation and bacterial species identification**

380   A PowerSoil DNA isolation kit (MoBio, Carlsbad, USA) was used for the isolation of bacterial

381   total genomic DNA extracted from sludge samples (1 mL) following the manufacturer's

382   protocol. Bacterial community composition was characterised by amplifying and sequencing

383   bacterial 16S ribosomal RNA (rRNA) gene fragments with the universal 16S PCR forward

384   primer (5'-

385   **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG-3')

386   and 16S PCR reverse primer (5'-

387   **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAA

388   TCC-3') following a standard protocol (67); nucleotide bases in bold are Illumina overhang

389   adapter sequences for high throughput sequencing. Amplified PCR products were purified

390   using an AMPure XP beads kit (Beckman Coulter Inc.) and concentrations recorded using a

391   Qubit® dsDNA HS Assay Kit (Life Technologies) according to the manufacturer's instructions

392   and then sequenced using an Illumina MiSeq machine. The resulting paired-end read DNA

393   sequence data were merged and quality filtered using the USEARCH sequence analysis tool

394   (68). Data were dereplicated so that only one copy of each sequence was reported. Sequence

395   data were then checked for chimeric sequences and clustered into groups of operational

396   taxonomic units based on a sequence identity threshold equal to or greater than 97% (thereafter

397   referred to as 97% OTUs) using the clustering pipeline UPARSE (68) in QIIME v.1.6.0, as

398   described in Bates et al. (69). Prokaryote phylotypes were classified to their corresponding

399   taxonomy by implementing the RDP classifier routine (70) in QIIME v. 1.6.0 (71) to

400   interrogate the Greengenes 13˙8 database (72). All sequences of chloroplast and mitochondrial

401   DNA were removed. Finally, DNA sequence data were rarefied to a depth of 5,600 randomly

402   selected reads per sample and two samples per treatment to achieve a standard number of

403   sequencing reads across all samples.

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405   **Statistical analyses**

406   Enzyme activities were plotted using the heat map function within the R package 'gplots'.

407   Similarities in bacterial community composition, enzyme activity and OMP remaining in

408   cultures were investigated using multivariate statistical analyses. Bray-Curtis distance matrices

409   of relative bacterial abundance, enzyme synthesis and OMP residual concentrations were

410   calculated and significant differences across all sample groups assessed using permutational

411   multivariate analysis of variance (PERMANOVA). The RELATE function within the

412   PRIMER package was used to calculate the Spearman rank correlation between data matrices

413   constructed from the bacterial community, enzyme activity and OMP residual concentrations.

414   All multivariate statistical analyses were performed in PRIMER 6 (Plymouth Marine

415   Laboratory). Significant differences in OMP removal and residual concentrations were

416   calculated by GraphPad Prism 7 using one-way ANOVA and post-hoc Tukey tests.

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419   **Acknowledgements**
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Table 1. Dissolved oxygen (DO) conditions used for the OMP removal studies. Controls had constant (unperturbed; 0 cycles/hr) DO supply at either high or low concentrations.

Operational condition	Dissolved Oxygen range (mg/L)	Perturbation frequency (cycles/hr)	Dissolved Oxygen shock durations (min.)	
			Oxygenation duration (min.)	Deoxygenation duration (min.)
High-DO Aerobic	8-9	0	Constant	Constant
	0-10	0.25	30	210
	4-6	1	10	50
	6-8	2	8	22
Low-DO Aerobic	2-3	0	Constant	Constant
	0-3	0.16	6	354
	0-4	0.25	6	234
	2-5	0.5	12	106

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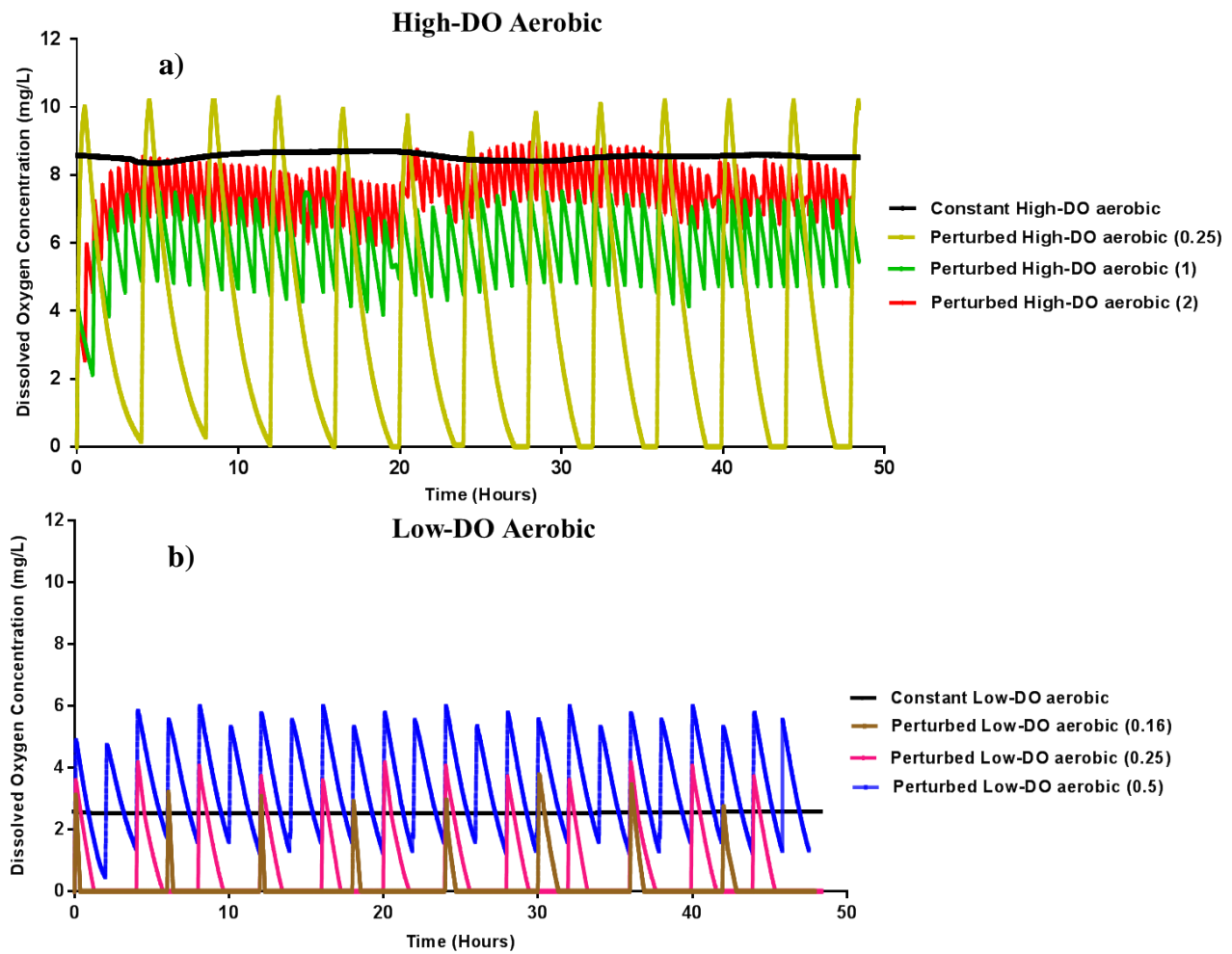
Figure 1. Dissolved oxygen profiles of microbial mixed cultures under: (a) high-DO and (b) low DO perturbation in comparison to non-perturbed conditions (constant). Stable DO profiles were achieved in the reactors that were provided constant aerobic (high and low) DO conditions, while aerobic-anaerobic DO transitions were induced with perturbed conditions.
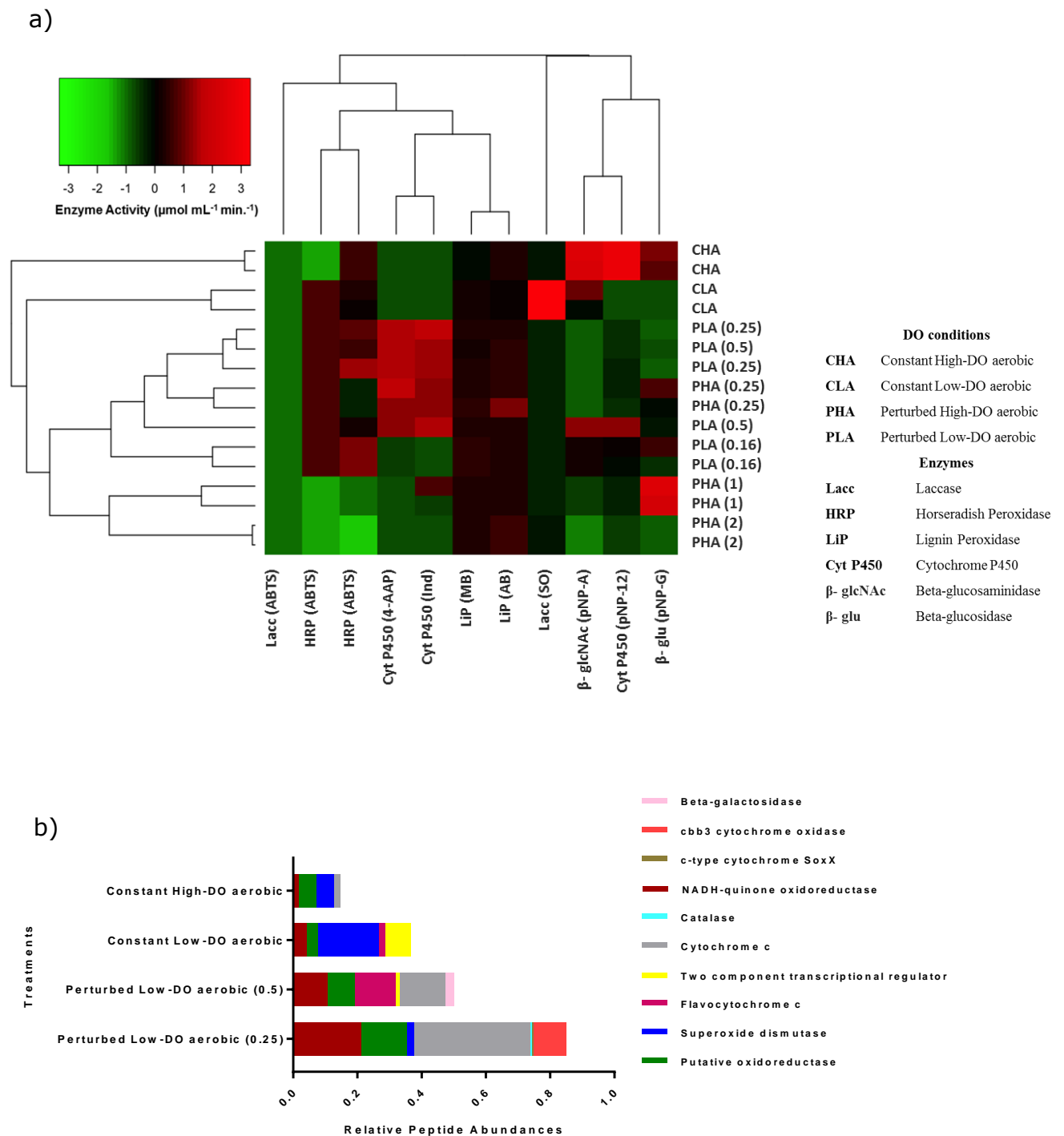
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682   **Figure 2. (a)** Oxidoreductase activities in: (a) DO perturbed and non-perturbed samples, and (b) relative peptide abundances
683   of expressed oxidoreductases (95 % confidence interval). Different rows represent DO perturbed frequencies in high and low-
684   DO regime; different columns represent expressed oxidoreductase activities. The color intensity in each panel shows the auto-
685   scaled enzyme activity (μmol mL^{-1} min.$^{-1}$) (a) across the gradient from red (highest enzyme activity) to green (lowest enzyme
686   activity).

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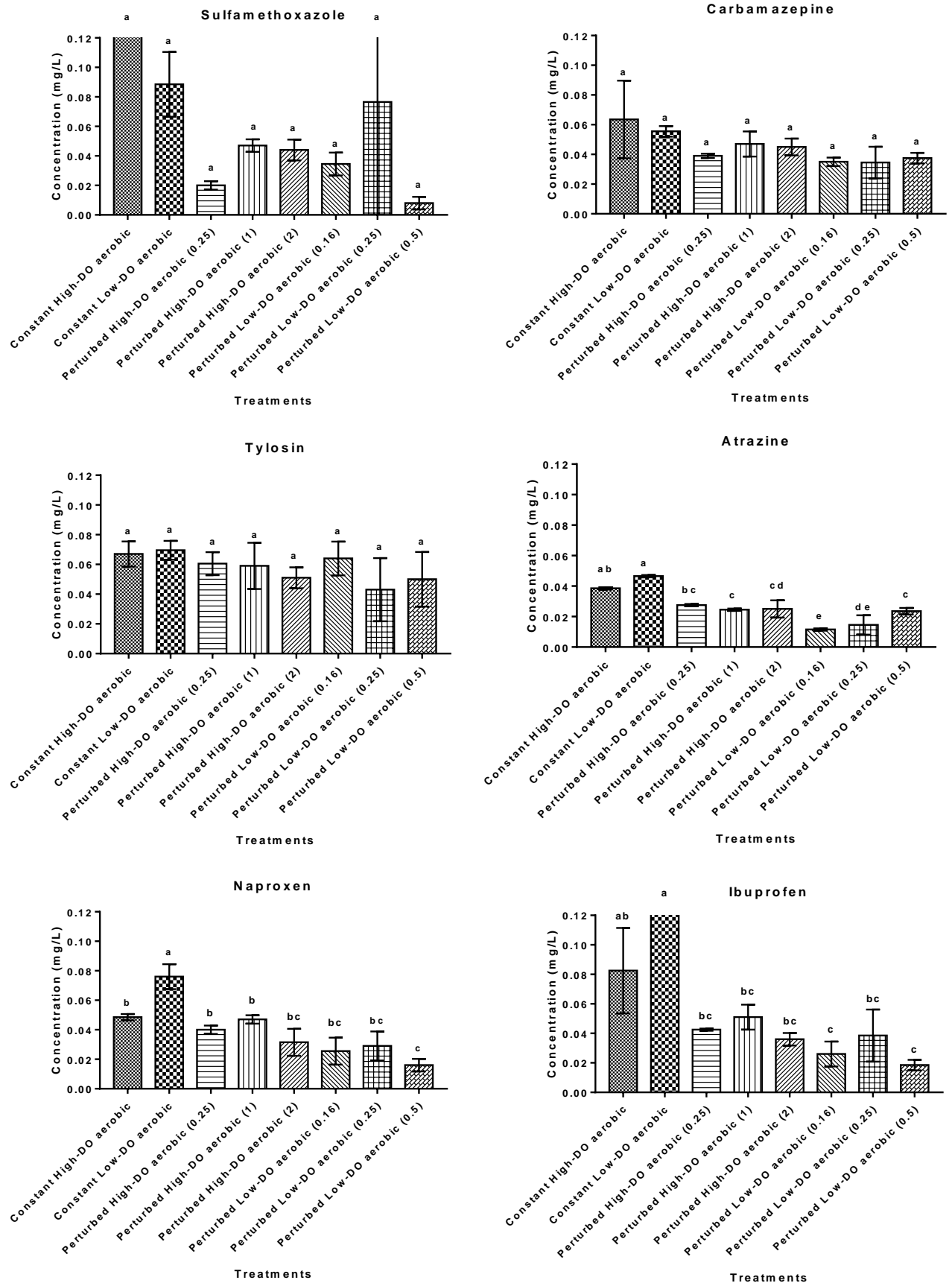
Figure 3. Residual concentrations of OMPs in supply-perturbed or constant supply (control) aerobic cultures at either high (8-9 mg/L) or low (2-3 mg/L) DO concentrations. Different letters above bars in the same graph indicate significant statistical differences between datasets via ANOVA (p = 0.05) and post hoc Tukey tests; bars indicated with the same letter are not significantly different.
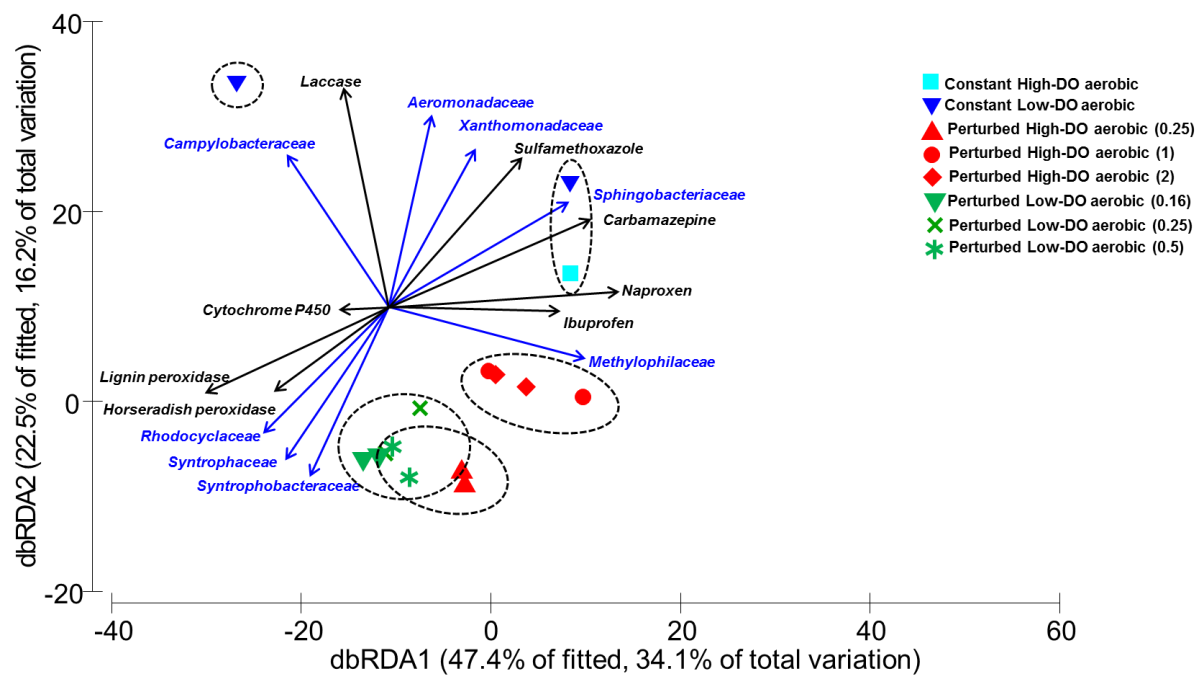
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737 Figure 4. Variation in bacterial community composition (Bray-Curtis similarity) as revealed by distance-based redundancy analysis
738 (dbRDA) of the data, illustrating relationships between bacterial families, enzyme activity and OMPs concentrations remaining in
739 DO perturbed and non-perturbed control cultures at different frequencies. The dbRDA was constrained by the best fitting
740 explanatory variables from multivariate multiple regression analysis (DISTLM) and vectors are shown for predictor variables
741 explaining significant proportions of bacterial community variation (p = 0.001) at 97% OTU richness.
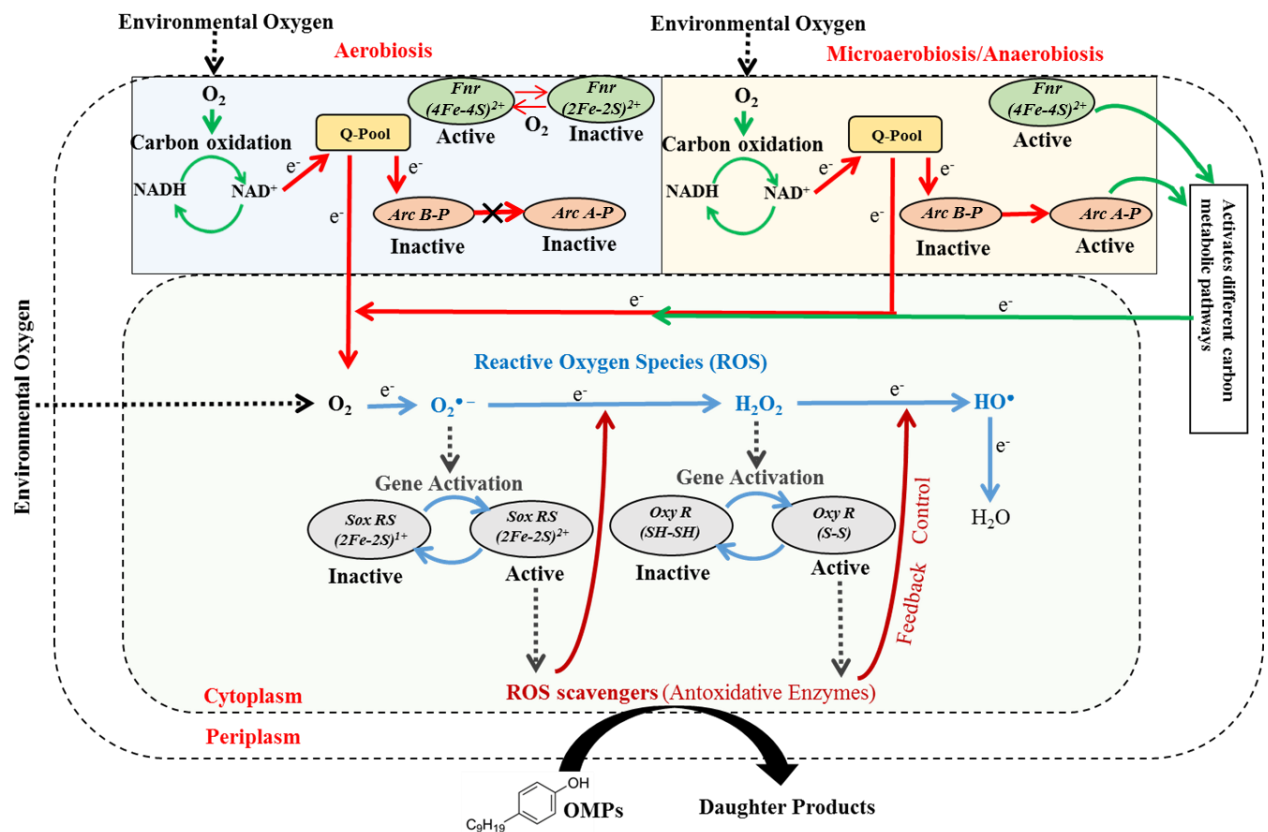
Figure 5. Regulation of enzyme production by the competition of ROS production and cellular respiration for O_2. Under aerobiosis, NADH generated by carbon oxidation is oxidised to NAD^+ and the electrons are passed on to oxygen by quinone pool (Q-pool). The autophosphorylation of membrane integrated Arc B is inhibited by oxidised quinone. Microaerobiosis/ Anaerobiosis causes a decrease in the level of oxidised quinones due to more production of NADH, allowing the transphosphorylation of Arc A-P. Arc A-P then coordinates the cellular response to microaerobiosis. Fnr is a direct sensor of oxygen availability. It is in a monomeric activated state with the incorporation of $(4Fe-4S)^{2+}$ cluster. Under aerobic conditions, the active Fnr $(4Fe-4S)^{2+}$ cluster gets converted to inactive $(2Fe-2S)^{2+}$, thus, inhibiting the gene regulation while microaerobic/ anaerobic conditions block the Fnr at active $(4Fe-4S)^{2+}$ form. Both Fnr and Arc AB regulates the cell metabolic pathways to generate redox signals which transfers electrons to the oxygen and generates the reactive oxygen species inside the cell. Another oxidative stress regulator (Sox RS and Oxy R) regulates the endogenous free radical concentrations by synthesis of antioxidative enzymes which simultaneously oxidise the organic micropollutants.