1 **Supporting Information**

2

3 **Induction of microbial oxidative stress as a new strategy to enhance the enzymatic**
4 **degradation of organic micropollutants in wastewater**

5 Amrita Bains[1], Octavio Perez-Garcia[1], Gavin Lear[2], David Greenwood[2], Simon Swift[3], Martin
6 Middleditch[2,] Edward Kolodziej[4,5,6], Naresh Singhal[1*]

7

8 *[1]Department of Civil and Environmental Engineering, University of Auckland, New*
9 *Zealand;[2]School of Biological Sciences, University of Auckland, New Zealand;[3]Faculty of*
10 *Medical and Health Sciences, University of Auckland, New Zealand;[4]Division of Sciences and*
11 *Mathematics, University of Washington-Tacoma; [5]Department of Civil & Environmental*
12 *Engineering, University of Washington; [6]Centre for Urban Waters, Tacoma, WA, USA.*

13

14 **Corresponding Author: Naresh Singhal. Department of Civil and Environmental*
15 *Engineering, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand.*
16 *Phone: +64 9 923 4512; Fax: +64 9 373 7462; Email: n.singhal@auckland.ac.nz*

17

18

SUPPLEMENTARY METHODS

Reactor operational parameters and sampling

The mixed liquor sludge from a dairy farm was washed three times by mixing and decanting 0.5 L of supernatant with distilled water (Milli-Q system, Millipore, Darmstadt, Germany) in volumetric cylinders to achieve volatile suspended solids (VSS) of 3 g-VSS/L. The synthetic wastewater feed consisted of sodium acetate trihydrate (63 mM), magnesium sulphate heptahydrate (3.6 mM), potassium chloride (4.7 mM), ammonium chloride (35.4 mM), di-potassium hydrogen phosphate (4.2 mM), potassium dihydrogen phosphate (2.1 mM) and 10 mL/L of a trace element solution (73), all purchased from Sigma Aldrich; purity ≥ 98% (St. Louis, MO, USA). The model OMPs were as follows: the veterinary and human antibiotics sulfamethoxazole (SMX) and tylosin (TYL); the pharmaceuticals carbamazepine (CBZ), ibuprofen (IBP) and naproxen (NPX) and the agrochemical atrazine (ATZ); all of purity ≥ 98% and purchased from Sigma Aldrich (St. Louis, MO, USA). The stock (1 g/L) for the model OMPs was prepared in methanol (Merck, Darmstadt, Germany). Oxygen (99%) was purchased from BOC (Auckland, New Zealand).

Enzyme activity assays

Oxidoreductases such as lignin peroxidase, horseradish peroxidase, laccase (derived from cultures of *Trametes versicolor*), beta-glucosidase (from *Aspergillus niger*) and cytochrome P450 (from human 3A4 isozyme microsomes), as well as the respective enzyme substrates (Methylene Blue, Azure B, L-DOPA (3,4-Dihydroxy-L-phenylalanine), ABTS (2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)), Sudan Orange G, pNP-A (4-nitrophenyl N-acetyl-β-D-glucosaminide), pNP-G (4-nitrophenyl- β-D-glucopyranoside), pNP-12 (4-nitrophenyl-dodecanoate), Indole and 4-AAP (4-Aminoantipyrine)) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium acetate trihydrate, glacial acetic acid (≥ 99% purity), di-potassium hydrogen phosphate, potassium di-hydrogen phosphate, dextrose and magnesium chloride hexahydrate of ≥ 99% purity were also obtained from Sigma Aldrich and used to prepare enzyme buffers at pH-5 and pH-7 respectively. The targeted oxidoreductases and their colorimetric probes are illustrated in Table S1. Two different buffers: 50 mM acetate buffer (50 mM sodium acetate trihydrate adjusted to pH-5 with glacial acetic acid) and 100 mM phosphate buffer (80 mM di-potassium hydrogen phosphate, 20 mM potassium dihydrogen phosphate, 10 mM dextrose, 6 mM magnesium acetate adjusted to pH-7.4) were used for the enzyme assays.

Protein extraction and identification

The high purity (≥ 99% purity) chemicals sodium chloride, tris-HCl, urea, thiourea, CHAPS, EDTA, dithiothreitol, Pefabloc SC, Pefabloc protector, trichloroacetic acid, triton-X, IPG, 2X Laemmlli buffers, Coomassie blue dye, iodoacetamide, ammonium bicarbonate, and trichloroacetic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Protein extraction was conducted for 2 days starting with a cell lysis phase followed by precipitation of proteins and separation of low and high stringency protein fractions by 1D SDS PAGE. On the first day, 30 ml of sludge sample was centrifuged at 20,000 xg for 20 min. The pellets were washed in 50 ml of 0.9% sodium chloride and spun down at 20,000 xg for 20 min at 4°C. Pellets were washed in 40 ml Tris-HCl (pH 7) and again pelleted down at 20,000 xg for 20 min at 4°C. The final pellets were resuspended in sample buffer following the recipe: 7M urea, 2M thiourea, 4% (w/v) CHAPS, 10 mM Tris-1 mM EDTA, 50 mM dithiothreitol, 25 mM Pefabloc SC and 2 mM Pefabloc protector and pulse-vortexed then placed on ice for 2 hours with regular mixing at 15 min intervals. The samples were sonicated for 15 sec. (6 rounds on ice) and centrifuged at 20,000 xg for 3 min at 4°C. Trichloroacetic acid (TCA: 100% (w/v)) was added to the supernatant so that the TCA concentration came to 10-20% and the samples incubated at -20 °C overnight. On the second day, the samples were centrifuged at 20,000 xg for 30 min at -4°C and the resulting pellet was washed with 5 ml cold acetone twice. The final pellet was heat dried to drive off acetone and then re-suspended by vortex mixing for 2 h in 400 µl low stringency buffer, comprising: 9M urea, 1% (v/v) Triton X-100, 1% (v/v) IPG buffer, 0.5% (w/v) dithiothreitol and a trace of bromophenol blue. The re-suspended samples were centrifuged at 20,000 xg for 45 min. resulting in the generation of a low stringency fraction (LSF) in the supernatant. The remaining protein pellet was re-suspended by vortex mixing for 2 hours in 400 µl high stringency buffer, comprising: 7M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) IPG buffer, 2% (w/v) dithiothreitol and a trace of bromophenol blue. The re-suspended samples were again centrifuged at 20,000 xg for 45 min resulting in the generation of a high stringency fraction (HSF) in the supernatant. Both fractions were quantified spectrophotometrically by fluorescence. Provisional separation of proteins was achieved using 1D PAGE. Briefly, sample preparation was carried out by dissolving pellets in 10 µl of MilliQ water and 10 µl of 2 X Laemmlli buffer and heating the mixture at 96°C for 2-3 minutes. The mixture was then cooled, centrifuged briefly and loaded onto a 4-12% SDS-PAGE gel. After running both LSF and HSF fractions on the gel and staining with Coomassie blue, zonal bands in the range of 250-350 kDa were each excised with a sharp razor blade and placed into low-binding, siliconised microcentrifuge tubes for destaining reduction, alkylation and finally

86 trypsinolysis. Then 5 µL of the generated tryptic peptides of the microbial proteins was injected
87 on a SCIEX 6600 triple TOF mass spectrometer. Protein identification was done by comparing
88 the obtained peptide sequences against those of the UniProt database.

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

90 A PowerSoil DNA isolation kit (MoBio, Carlsbad, USA) was used for the isolation of bacterial
91 total genomic DNA extracted from sludge samples (1 mL) following the manufacturer's
92 protocol. All the extractions were performed in duplicate. Bacterial community composition
93 was characterised by amplifying and sequencing a fragment of the bacterial 16S ribosomal
94 RNA (rRNA) gene following a standard protocol (Illumina 2013). The V3 and V4 region of
95 16S rRNA genes were amplified from individual DNA extracts with the universal 16S
96 Amplicon PCR Forward Primer (5'-
97 **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG-
98 3') and 16S Amplicon PCR Reverse Primer (5'-
99 **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAA
100 TCC-3') (67). These primers have been validated to provide good bacterial phylum coverage
101 as they are also modified to include Illumina adapter overhang sequences (in bold) required for
102 downstream DNA sequencing. DNA amplification was conducted as follows: (i) 94˚C for 3
103 min; (ii) 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec; (iii) 72˚C for 5 min.
104 Following amplification, PCR products were purified using the AMPure XP beads kit
105 (Beckman Coulter Inc., Brea, CA, USA) according to the manufacturer's instructions. The
106 concentrations of purified amplicons were finally measured and recorded using a Qubit®
107 dsDNA HS Assay Kit (Life technologies, Carlsbad, CA, USA) and submitted to New Zealand
108 Genomics Ltd for sequencing by Illumina MiSeq machine. The resulting paired-end read DNA
109 sequence data were merged and quality filtered using the USEARCH sequence analysis tool
110 (68). Data were dereplicated so that only one copy of each sequence was reported, and
111 'singleton' sequences represented by only one DNA sequence in the database were removed.
112 Sequence data were then checked for chimeric sequences and clustered into groups of
113 operational taxonomic units based on a sequence identity threshold equal to or greater than
114 97% (thereafter referred to as 97% OTUs) using the clustering pipeline UPARSE (68) in
115 QIIME v.1.6.0 as described in (69). After that, prokaryote phylotypes were classified to their
116 corresponding taxonomy by implementing the RDP classifier routine (70) in QIIME v. 1.6.0
117 (71) to interrogate the Greengenes 13˙8 database (72). All sequences of chloroplast and
118 mitochondrial DNA were removed. Finally, DNA sequence data were rarefied to a depth of

119 5,600 randomly selected reads per sample and two samples per treatment to achieve a standard
120 sequencing reads across all samples.

1. Oxidoreductases and substrate dyes

Table S1. Target oxidoreductases and the dyes used to detect their activity in respective buffers.

Target enzyme	Enzyme substrate (Dye)	Solution buffer
Lignin peroxidase	Methylene Blue	acetate buffer
(LiP)	Azure B	acetate buffer
Horseradish peroxidase	L-DOPA	acetate buffer
(HRP)	ABTS	acetate buffer
Laccase	Sudan Orange	acetate buffer
(Lac)	ABTS	acetate buffer
β-glucosaminidase (β-glcNAc)	pNP-A	acetate buffer
β-glucosidase (β-glu)	pNP-G	acetate buffer
Cytochrome P450	pNP-12	phosphate buffer
(Cyp450 or CYPcam)	Indole	phosphate buffer
	4-AAP	phosphate buffer

2. OMP concentrations in the bioreactors and statistical analysis

Table S2. Residual concentrations (mg/L) of OMPs under constant non-perturbed and perturbed DO frequencies (0.16, 0.25, 0.5, 1 and 2 cycles/hr) in different aeration regimes (high and low-DO) measured by LC-MS. Statistical differences among the samples are indicated with $p<0.05$ performed on duplicate sample set ($n = 2$) as mean ± standard deviation.

Compounds	Constant High Aerobic	Constant Low Aerobic	Perturbed High Aerobic (0.25)	Perturbed High Aerobic (1)	Perturbed High Aerobic (2)	Perturbed Low Aerobic (0.16)	Perturbed Low Aerobic (0.25)	Perturbed Low Aerobic (0.5)
Sulfamethoxazole	0.12 ± 0.02	0.09 ± 0.03	0.02 ± 0.00	0.05 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.07 ± 0.07	0.01 ± 0.00
Carbamazepine	0.06 ± 0.02	0.06 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.05 ± 0.06	0.04 ± 0.00
Tylosin	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.1 ± 0.1	0.05 ± 0.01
Atrazine	0.05 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.04 ± 0.05	0.02 ± 0.00
Naproxen	0.08 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.03 ± 0.01	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.00
Ibuprofen	0.12 ± 0.03	0.08 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.03 ± 0.02	0.04 ± 0.01	0.02 ± 0.00

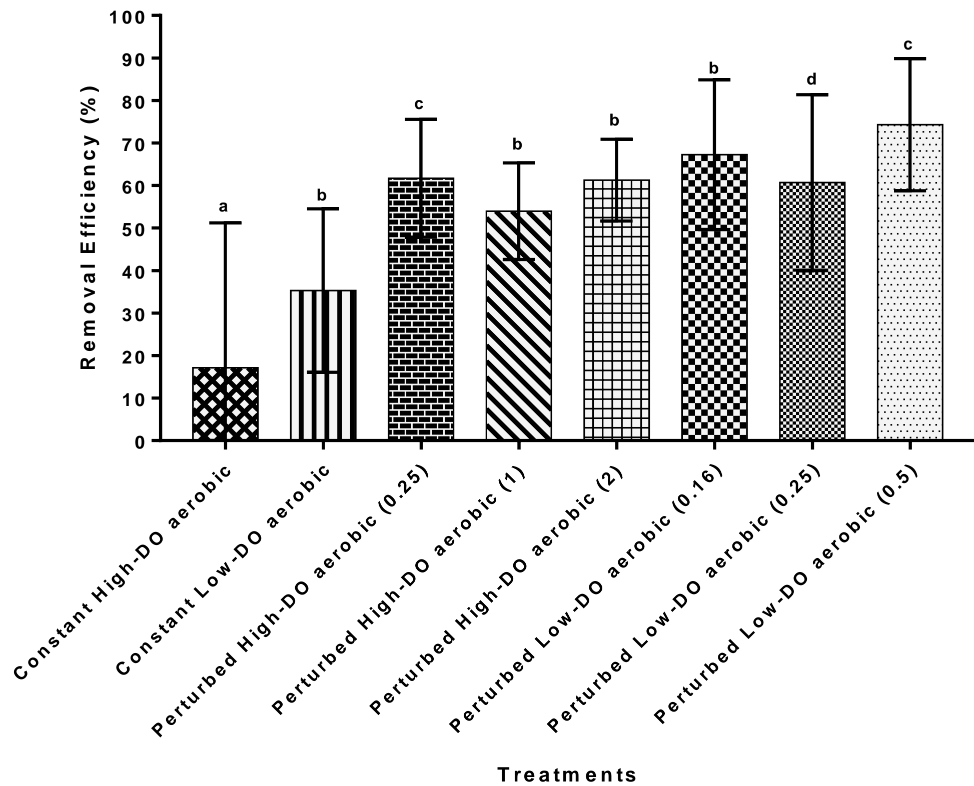
151

Table S3. Quality control parameters (Recovery percentage, Limit of detection (LOD) and Limit of quantification (LOQ)) of OMP extraction and analysis methods used. The LOD (mg/L) and LOQ (mg/L) are represented as mean ± standard deviation.

Compounds	Recovery (%)	Limit of Detection (LOD)	Limit of Quantification (LOQ)
Sulfamethoxazole	95 ± 2.2	0.060	0.181
Carbamazepine	100 ± 5.6	0.152	0.461
Tylosin	54 ± 2.5	0.095	0.288
Atrazine	100 ± 1.7	0.010	0.031
Naproxen	80 ± 2.4	0.117	0.354
Ibuprofen	100 ± 7.9	0.049	0.150

152 153 ## 3. Figure S1. Removal efficiency of OMPs form bioreactor culture at different aeration regimes

154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169



170 171 172 173 174 175 **Figure S1.** OMP removal efficiency was significantly increased with DO perturbation, as compared to within non-perturbed cultures ($p < 0.05$). Cultures under the perturbed low-DO aerobic regime (0.16, 0.25 and 0.5 cycles/hr frequencies) showed more OMP removal than cultures treated under perturbed high-DO aerobic (frequencies 1-2 cycles/hr) and non-perturbed constant high and low-DO aerobic regimes, respectively ($n = 2$). Different letters above bars denote significant differences between datasets according to post hoc Tukey tests at $p = 0.05$, treatments indicated with same letters are not statistically different.

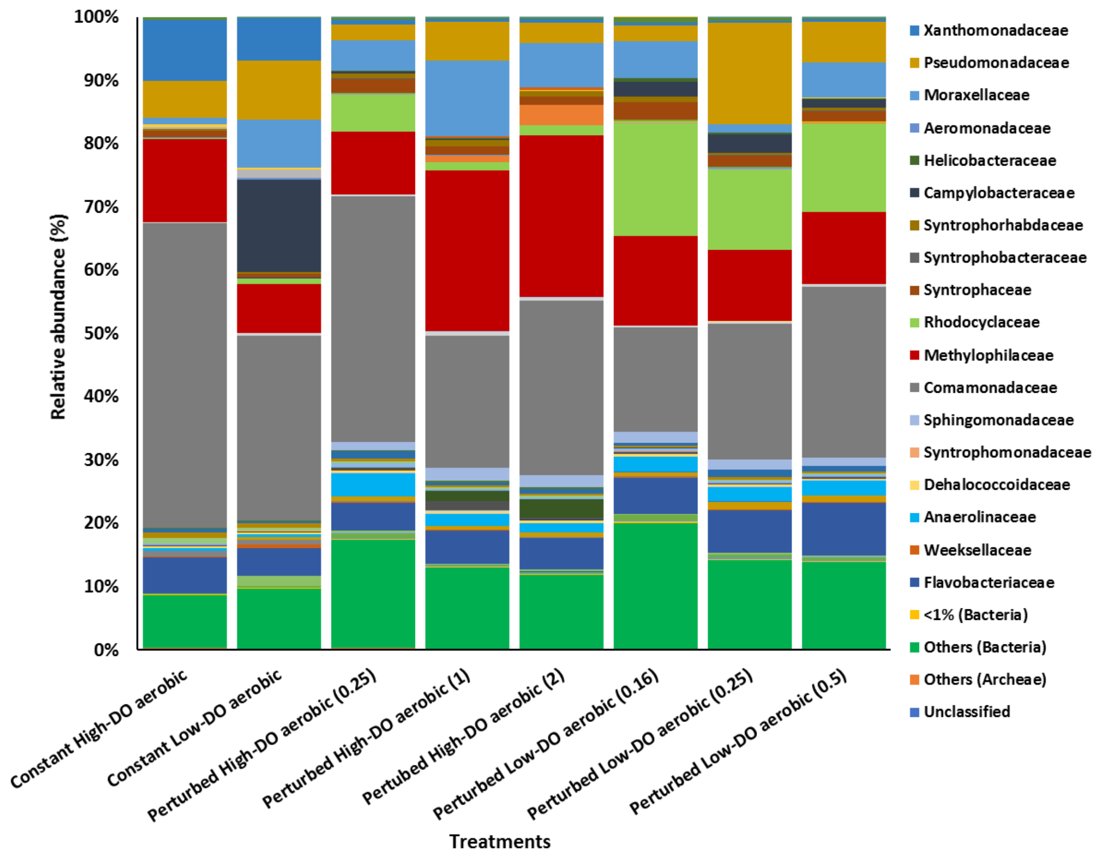
176

177 **4. Figure S2. Microbial Speciation with 16S rRNA gene sequencing**

178

179

180



181 182 183 184 185 186 187 188 189 190 191 192 193 194

195 **Figure S2.** Composition of bacterial communities for taxa grouped at the family level in DO non-perturbed (constant) and
196 perturbed) cultures. All analyses were done in duplicate (n = 2).