

# Metabolomics reveals the physiological response of *Pseudomonas putida* KT2440 (UWC1) after pharmaceutical exposure

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1 **Metabolomics reveals the physiological response of *Pseudomonas***  
2 ***putida* KT2440 (UWC1) after pharmaceutical exposure.**

3

4 Running title: Metabolomics reveals abiotic perturbations to *P. putida*

5

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19

20

21 **Abstract**

22 Human pharmaceuticals have been detected in wastewater treatment plants, rivers, and  
23 estuaries throughout Europe and the United States. It is widely acknowledged that there  
24 is insufficient information available to determine whether prolonged exposure to low  
25 levels of these substances is having an impact on the microbial ecology in such  
26 environments. In this study we attempt to measure the effects of exposing cultures of  
27 *Pseudomonas putida* KT2440 (UWC1) to six pharmaceuticals by looking at differences  
28 in metabolite levels. Initially, we used Fourier transform infrared (FT-IR) spectroscopy  
29 coupled with multivariate analysis to discriminate between cell cultures exposed to  
30 different pharmaceuticals. This suggested that on exposure to propranolol there were  
31 significant changes in the lipid complement of *P. putida*. Metabolic profiling with gas  
32 chromatography-mass spectrometry (GC-MS), coupled with univariate statistical  
33 analyses, was used to identify endogenous metabolites contributing to discrimination  
34 between cells exposed to the six drugs. This approach suggested that the energy  
35 reserves of exposed cells were being expended and was particularly evident on exposure  
36 to propranolol. Adenosine triphosphate (ATP) concentrations were raised in *P. putida*  
37 exposed to propranolol. Increased energy requirements may be due to energy dependent  
38 efflux pumps being used to remove propranolol from the cell.

39

40 **Introduction**

41 Studies spanning the last 20 years, have shown that human pharmaceuticals are present  
42 in waste water treatment plants (WWTPs), rivers and estuaries at the  $\text{ng L}^{-1}$  or low  $\mu\text{g L}^{-1}$   
43 level throughout Europe and in the United States.<sup>1-9</sup> In monitoring studies in the UK 13  
44 pharmaceuticals selected from priority lists of the UK Environment Agency and the  
45 Oslo and Paris Commission (OSPAR) were detected at concentrations ranging from 11  
46 – 69,570  $\text{ng L}^{-1}$  in raw WWTP effluent<sup>1</sup>.

47 Wastewater from large industrial sites in India and China producing generic drugs in  
48 bulk for the global market has been shown to be a source of far higher environmental  
49 concentrations of pharmaceuticals<sup>10</sup>. Fick and colleagues showed contamination of  
50 surface, ground and drinking water in the Hyderabad drug-producing area, where 9  
51 drugs were detected in the  $\text{mg L}^{-1}$  range in two lakes and at high  $\text{ng L}^{-1}$  or low  $\mu\text{g L}^{-1}$   
52 levels in wells located in surrounding villages.<sup>11</sup> The effluent from the WWTP serving  
53 approximately 90 bulk drug manufacturers shown to contain high levels of drugs with a  
54 range of vertebrate drug targets was toxic to aquatic vertebrates even at high dilutions,  
55 with 40% reduced growth in tadpoles in diluted (0.2%) effluent, and a median lethal  
56 concentration for zebrafish between 2.7-8.1%.<sup>12</sup>

57 Pharmaceuticals merit concern as environmental pollutants because they are designed  
58 with high potency and high specificity for interaction with biological systems: they are  
59 of possible harm to the environment because they are designed to target specific  
60 receptors/enzymes, which may have homologs in other species. Although studies such  
61 as that of Gunnarsson *et al.*<sup>13</sup> conclude that bacteria have both low numbers of  
62 homologs and low sequence similarity to those in man, many microbial organisms have  
63 an important environmental role which includes element cycling and the degradation of  
64 xenobiotic pollutants, and stress responses due to chronic exposure might impact on

65 fitness for survival. While Sumpter in his recent review<sup>14</sup> concludes that the vast  
66 majority of pharmaceuticals will have no appreciable real-world environmental  
67 consequence it has been shown that pharmaceuticals have a range of acute and chronic  
68 toxicities on test organisms where environmental exposure is persistent at low  
69 concentrations.<sup>15, 16</sup> It has been suggested that both *concentration addition*, where  
70 multiple compounds of the same class are present, each at low concentration, and  
71 *response addition* where multiple compounds of different classes are present, may cause  
72 stress responses in organisms and impact on fitness for survival.<sup>17-20</sup> Environmental risk  
73 assessments (ERA) are now required when applying for marketing authorization for a  
74 medicinal product for human use in the European Union (EU) (Council Directive  
75 2001/83/EC as amended by Council Directive 2004/27/EC). In a tiered approach to  
76 testing, evaluation beyond acute toxicity tests is required where the predicted  
77 environmental concentration (PEC) of a pharmaceutically active compound is more than  
78 0.01 µg L<sup>-1</sup>. There is clearly a need to ascertain chronic effects of pharmaceuticals in the  
79 environment, and to mitigate pollution due to bulk drug manufacture.

80

81 Here, we studied the effects of pharmaceutical exposure on *Pseudomonas putida*, a  
82 metabolically versatile soil bacterium, able to undertake important metabolic activities  
83 in the environment, including element cycling and the degradation of xenobiotic  
84 pollutants.<sup>21</sup> *P. putida* strain KT2440 has been certified as a biosafety host for the  
85 cloning of foreign genes, and has potential for biotechnological applications such as  
86 bioremediation and biocatalysis.<sup>22</sup> *P. putida* KT2440 UWC1 is a plasmid-free,  
87 restriction-negative, spontaneous rifampin-resistant derivative of *P. putida* KT2440.<sup>23</sup>  
88 Recent sequencing of the 6.1Mb genome of *P. putida* KT2440<sup>24</sup> has revealed diverse

89 metabolic and transport systems, with a comparatively high number of efflux pumps  
90 associated with protection against toxic substrates and metabolites.

91 Metabolomics is a well-established field for the global analysis of endogenous  
92 metabolites within cells, tissue, biofluids, organs or whole organisms, and utilizes  
93 analytical techniques combined with statistical analysis. Where traditional studies may  
94 focus on one or a few biochemical pathways, discovery metabolomics attempts to  
95 observe global metabolite alterations. As such, discovery metabolomics is considered to  
96 be hypothesis generating, rather than hypothesis testing, with the possibility of finding  
97 novel results which should be tested further targeted experiments. Metabolomics offers  
98 several distinct advantages over other omic studies. A ten-fold difference is generally  
99 observed in the number of metabolites compared to the number of genes (fewer  
100 metabolites than genes), making the metabolome more amenable to both chemical  
101 analysis and data interpretation.<sup>25, 26</sup> While alterations in the transcriptome or proteome  
102 may not always lead to changes in the metabolic phenotype<sup>27</sup>, the metabolome  
103 represents the final products of gene expression and is closest to the function or  
104 phenotype of the cell. Furthermore, metabolic control analysis (MCA) has  
105 demonstrated that changes in concentration of metabolites can be observed even when  
106 alterations in the concentrations of transcripts and proteins are small.<sup>28</sup> Metabolomics is  
107 a high-throughput strategy with low costs per analysis compared to transcriptomic and  
108 proteomic technologies, and, unlike other omics techniques, does not rely on species-  
109 specific information. The reader is directed to the literature for information on the  
110 analytical technologies and methodologies<sup>29-36</sup>, and on the statistical approaches used in  
111 metabolomics.<sup>37</sup> Environmental metabolomics has recently been defined as the  
112 application of metabolomics techniques to characterise the metabolism of free living  
113 organisms obtained from the natural environment and of organisms reared under

114 laboratory conditions, where those conditions specifically serve to mimic scenarios  
115 encountered in the natural environment.<sup>38</sup> There is considerable potential for omic  
116 profiling methods to progress significant advances in regulatory ecotoxicology, with  
117 applications including biomarker development and risk assessment for toxicant  
118 exposure, and evaluation of metabolic responses to environmental stressors.<sup>39, 40</sup>  
119 Reviews of recent applications in environmental metabolomics can be found in<sup>41, 42</sup>.  
120 In whole organism fingerprinting FT-IR spectroscopy measures bond vibrations of  
121 functional chemical groups in cell constituents such as DNA and RNA, proteins, lipids  
122 and carbohydrates. FT-IR may be used as a screening tool providing rapid  
123 discrimination between samples, through measurement of overall phenotypic changes in  
124 a sample without specific identification of the individual metabolites responsible. In  
125 contrast, metabolic profiling by GC-MS provides semi-quantification and, where  
126 possible, the definitive identification of metabolites through retention time and mass  
127 spectrum matching. Subsequent data analysis can then reveal discriminatory  
128 metabolites.<sup>33</sup>

129

130 In this study we monitored the effect on the metabolism of *P. putida* KT2440 UWC1 of  
131 exposure to six pharmaceutical compounds; four analgesics (3 non-steroidal anti-  
132 inflammatory drugs (NSAID) and acetaminophen - a possible COX -isoform inhibitor  
133 but with an ill-defined mechanism of action), and two  $\beta$ -adrenergic receptor agonists  
134 (Table SI3 1, Supplementary Information). Five of the compounds acetaminophen,  
135 diclofenac, ibuprofen, mefenamic acid and propranolol have been detected in  
136 wastewater treatment plant effluent in the UK.<sup>1</sup> Acetaminophen was detected in raw  
137 effluent only at a mean concentration of 27,341 ng L<sup>-1</sup>, diclofenac throughout the  
138 treatment plant at concentrations ranging from 342-978 ng L<sup>-1</sup>, ibuprofen 3063-23,161

139 ng L<sup>-1</sup>, mefenamic acid 234-959 ng L<sup>-1</sup> and propranolol 83-291 ng L<sup>-1</sup>. Roberts and  
140 Thomas suggest that it would also be beneficial to determine levels of parent  
141 compounds present in sewage sludge, in order to determine levels of adsorption.<sup>1</sup>

142 In this study we exposed *P. putida* KT2440 to the pharmaceuticals at a single  
143 concentration of 50 µg mL<sup>-1</sup>; although higher than measured environmental  
144 concentrations in the UK, this is a concentration well below the minimum inhibitory  
145 concentrations established for the pharmaceuticals, and at which we had seen a  
146 measurable effect in earlier experiments using FT-IR spectroscopy. We performed a  
147 principal components-canonical variates analysis on the FT-IR spectra of whole cells,  
148 and ANOVA and correlation analysis on the GC-MS profiles of the methanol cell  
149 extracts of *P. putida* exposed either to one of the pharmaceuticals or to water as a  
150 control. Metabolic fingerprinting by FT-IR spectroscopy suggested that on exposure to  
151 propranolol there were significant changes in the lipid complement of *P. putida*.  
152 Metabolic profiling from GC-MS measurements suggested that the energy reserves of  
153 exposed cells were being expended and this was particularly evident on exposure to  
154 propranolol. Therefore we measured adenosine triphosphate (ATP) concentrations in *P.*  
155 *putida* exposed to propranolol using a bioluminescence assay.

156

## 157 **Materials and Methods**

158 **Materials and Methods for the experiment and for the statistical analysis** are  
159 described in detail in the Supplementary Information. In preliminary experiments the  
160 effect of each pharmaceutical on growth of *P. putida* was determined, the minimum  
161 inhibitory concentration (MIC) of the pharmaceuticals for *P. putida* KT2440 UWC1  
162 was estimated, and the recovery of the pharmaceuticals was monitored by HPLC in



163 order to assess if there was any metabolism of the pharmaceuticals by *P. putida* over 24  
164 h. For the metabolomics analysis, briefly, *Pseudomonas putida* KT2440 UWC1 was  
165 cultured, in replicate, in liquid medium supplemented with one of 6 drugs at a  
166 concentration of 50  $\mu\text{g mL}^{-1}$ , or water as a control. At the end of the exponential growth  
167 period cells were harvested and the sample split to provide cells for FT-IR spectroscopy  
168 and for GC-MS. Metabolite fingerprinting of whole cells by FT-IR spectroscopy was  
169 carried out according to a modified method of Goodacre *et al.*<sup>43</sup> Metabolite profiling of  
170 methanol cell extracts by GC-MS was carried out according to a modified method of  
171 Winder *et al.*<sup>30</sup> using GC-MS conditions optimized for yeast.<sup>44</sup> ATP in methanol:water  
172 extracts of *P. putida* exposed to propranolol was measured using a bioluminescence  
173 assay kit available from Roche Molecular Biochemicals (Roche Diagnostics, Burgess  
174 Hill UK). Full details are given in the supplementary material. A combined principal  
175 components-canonical variates analysis (PC-CVA) was carried out for both the FT-IR  
176 spectra and GC-MS data using programs written in MATLAB<sup>45</sup> as detailed elsewhere.<sup>46</sup>  
177 ANOVA was carried out on GC-MS data using programs written in MATLAB  
178 (<http://www.mathworks.com/>) and described elsewhere.<sup>37</sup> Correlation analysis for  
179 metabolomics data is described by Steuer.<sup>47, 48</sup> Correlation analysis for significantly  
180 altered metabolites was carried out using Graphviz open source graph visualization  
181 software<sup>49</sup> following an approach proposed by Kamada and Kawai.<sup>50</sup> Full details are  
182 given in the supplementary material.

## 183 **Results and Discussion**

184 Results for the determination of minimum inhibitory concentrations, the effect of each  
185 pharmaceutical on growth and monitoring recovery of the pharmaceuticals by HPLC are  
186 given in the Supplementary Information.

187 *Multivariate Analysis of FT-IR Data:* A total of 26 PCs were extracted for a cross-  
188 validated PC-CVA model for the FT-IR spectra. Figure 1a shows the PC-CV score 1  
189 plotted against PC-CV score 2 for the FT-IR spectra of *P. putida* exposed to the six  
190 pharmaceuticals. In this analysis, the FT-IR spectra from 4 replicate cultures of *P.*  
191 *putida* were used as a training set, and the spectra from the fifth replicate were used as  
192 an independent test set with no *a priori* knowledge of the class structure. The test data  
193 should lie within the bounds of the training data, defined here as the 95% confidence  
194 limit from the group centres here constructed around each group mean by the  $\chi^2$   
195 distribution on two degrees of freedom, as observed for *P. putida* exposed to ibuprofen  
196 and mefenamic acid. The cells exposed to propranolol and ibuprofen are separated, both  
197 from the control and cells exposed to the remaining pharmaceuticals, along PC-CV1,  
198 and those exposed to mefenamic acid along PC-CV2. No effect on *P. putida* exposed to  
199 acetaminophen, atenolol or diclofenac was observed on inspection of the lower  
200 canonical variates (data not shown). Examination of the loadings for PC-CV1 from the  
201 PC-CV analysis (Figure 1b) shows, firstly, significantly high loadings occurring at  
202 several wavenumbers for propranolol at 1570, 1483, 1271, 1242, and 1102  $\text{cm}^{-1}$ . High  
203 loadings in the region corresponding to aliphatic C-H, and hence bacterial fatty acids, at  
204 2919 and 2850  $\text{cm}^{-1}$  prompted us to investigate lipid alterations in *P. putida* exposed to  
205 propranolol. There are also significantly high loadings in the regions corresponding to  
206 the amide I bands in protein structures at 1655 $\text{cm}^{-1}$  ( $\alpha$ -helical structures), 1709, 1659  
207 and 1630  $\text{cm}^{-1}$  ( $\beta$ -sheet structures).<sup>51</sup>

208 These observations, together with the reduction in free amino acids observed in the  
209 GC-MS analysis (*vide infra*) are consistent with the theory that cell integrity is  
210 maintained through *cis-to-trans* isomerization of membrane lipids which results in a

211 more rigid cell membrane structure, and synthesis of drug efflux pumps in order to  
212 remove toxic substances from the cell.<sup>52</sup>  
213 The PC-CVA was repeated using absorbances at wavenumbers selected from those with  
214 significantly high loadings shown in Figure 1b. Rebuilding the model with absorbances  
215 only at wavenumbers significant for bacterial fatty acids showed discrimination between  
216 *P. putida* exposed to propranolol and the control, and no discrimination of any other  
217 exposure from the control (Figure 2). Thus, observed lipid alterations were specific for  
218 exposure to propranolol. PC-CVA models built using absorbances at selected  
219 wavenumbers significant for the fingerprint region showed little difference from the  
220 model using the entire dataset, affording no new information, while there were too few  
221 wavenumbers significant for protein to use successfully in a PC-CVA model.

222

223 (Figure 1)

224 (Figure 2)

225

226 *Univariate Analysis of GC-MS Data:* ANOVA was performed for *P. putida* exposed to  
227 each pharmaceutical versus the control using the family-wise error rate (FWER) to  
228 determine a suitable threshold for the p-value.<sup>53</sup> Thresholds equivalent to  $\alpha = 0.05$  were  
229 determined for cells exposed to propranolol (0.0177), diclofenac (0.006),  
230 acetaminophen (0.005), atenolol (0.003) and mefenamic acid ( $1.76 \times 10^{-4}$ ). A threshold  
231 was determined equivalent to  $\alpha = 0.1$  for cells exposed to ibuprofen (0.013), in order to  
232 be able to compare alterations in metabolites, since discrimination of these samples was  
233 earlier observed from the FT-IR data. A total of 76 metabolites were significantly  
234 altered overall with p-values below the FWER thresholds, and 67 of these had an area  
235 under the ROC curve<sup>54</sup>  $> 0.85$ . Of these, 43 were altered on exposure to propranolol, 17

236 on exposure to diclofenac, 16 on exposure to ibuprofen, 14 on exposure to  
237 acetaminophen, 8 on exposure to atenolol, and 3 on exposure to mefenamic acid.  
238 Metabolites are listed in the supporting information (Table SI2 1.), together with the p-  
239 value and fold difference in median GC-MS peak response. In order to view alterations  
240 which are common to exposure to the different pharmaceuticals, the 67 metabolites are  
241 ordered firstly by significance (p-value) for exposure to propranolol, followed by  
242 significance for exposure to diclofenac, ibuprofen, acetaminophen etc.

243 Metabolite identification is currently recognised as a major limitation in GC-MS  
244 metabolomics studies, and a number of studies report metabolites of biological interest  
245 as unidentified.<sup>55</sup> The accurate identification of metabolites requires the construction of  
246 mass spectral / retention index libraries. Commercially available GC-MS libraries such  
247 as NIST/EPA/NIH and Wiley have not been developed with the objective of including  
248 endogenous (or exogenous) metabolites, and are not widely applicable in metabolomics  
249 studies. A number of research groups have, therefore, developed their own metabolite  
250 libraries employing both the mass spectrum and retention index to define a metabolite,  
251 for example the Golm (<http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html>),<sup>56</sup> and  
252 Feihn (<http://fiehnlab.ucdavis.edu/Metabolite-Library-2007/>);<sup>57</sup> databases. For definitive  
253 metabolite identification, comparison of fragmentation mass spectra with authentic  
254 chemical standards is required. Standards are often unavailable for endogenous  
255 metabolites and therefore only possible or probable identifications can be assigned in a  
256 large number of instances. In this study those metabolites where the identification is  
257 definitive are labeled with the metabolite name and an asterisk in the supplementary  
258 information, those where the identification is tentative labeled with the metabolite  
259 name, while unidentified metabolites are identified with a number. The recorded fold

260 difference is the median fold difference in GC-MS peak response (cells exposed to a  
261 pharmaceutical / control cells exposed to water).

262 Altered metabolites in *P. putida* exposed to propranolol are visualised in Figure 3,  
263 where the area under the ROC curve is plotted *versus* the p-value from the ANOVA  
264 analysis. Metabolites with an area under the ROC curve  $> 0.7$  and a p-value  $< 2 \times 10^{-2}$   
265 are labeled, and metabolites with an area under the ROC curve  $> 0.85$ , with  $p < 0.01$   
266 were considered significant and selected for correlation analysis.

267 (Figure 3)

268 (Figure 4)

269 Spring-embedded correlation plots for the significant metabolites identified from the  
270 ANOVA analysis show correlations in the cells exposed to water (Figure 4a) and  
271 correlations in the cells exposed to propranolol (Figure 4b). Correlation analyses for *P.*  
272 *putida* exposed to the other pharmaceuticals were not as informative as that for  
273 propranolol and are not shown; for example in *P. putida* exposed to ibuprofen  
274 correlations were observed only between trehalose and metabolites 35 and 47, and  
275 further to 2-aminoethyldihydrogenphosphate and tropic acid, and between cellobiose,  
276 putrescine, glycerol-3-phosphate a sugar alcohol and metabolite 95.

277

278 In *P. putida* exposed to propranolol 9 metabolites identified with the low p-values in the  
279 ANOVA analysis ( $< 5.6 \times 10^{-4}$ ) were raised in concentration from, or lowered in  
280 concentration to, near the analytical limit of detection in exposed cells (Figure SI3 2 in  
281 the supplementary information). All except metabolites 130 and 131 had an area under  
282 the ROC curve of 1, implying that these metabolites are entirely diagnostic of cells  
283 exposed to propranolol. Propranolol itself was present in the extracts of exposed cells  
284 and absent in the control cells. The concentration of metabolites 129 and 130 (for which

285 there was no definitive identification) fell below the limit of detection in exposed cells.  
286 Metabolite 129 was also altered significantly in cells exposed to diclofenac and  
287 mefenamic acid. Metabolites 131, 134, 135, 139, 142, and 145 were raised in exposed  
288 cells from near or below the limit of detection in the control, and were not present in  
289 cells exposed to any other pharmaceutical in the study. Identification was not possible  
290 from the mass spectra which contained only low m/z ions. Further work using <sup>13</sup>C-  
291 labeled propranolol would determine whether propranolol is metabolized by *P. putida*,  
292 and whether metabolites 139, 134, 135, 142 and 145 are endogenous metabolites or  
293 products of propranolol catabolism.

294

295 In *P. putida* exposed only to water, metabolite 130 is correlated with the amino acid  
296 valine, and correlation extends through other amino acids to trehalose and cellobiose.  
297 This correlation is disrupted within the sample set of *P. putida* exposed to propranolol  
298 as the concentration of metabolite 130 falls to near to the limit of detection. In cells  
299 exposed to propranolol the concentrations of trehalose and cellobiose are lowered by 0.5  
300 and 0.8, respectively, when compared to *P. putida* exposed only to water. In *P. putida*  
301 exposed to propranolol, propranolol itself is correlated to both trehalose and cellobiose  
302 and another energy related metabolite, a sugar phosphate, and correlation is extended  
303 from propranolol through cellobiose and metabolite 139 to succinic acid, a metabolite of  
304 the TCA cycle. Concentrations of sugar phosphates and succinic acid were raised, 1.3 -  
305 2.1 fold and 3 fold, respectively, in *P. putida* exposed to propranolol, suggesting  
306 increased glucose flux through glycolysis, as does utilization of trehalose and cellobiose  
307 (Figure 5).

308

(Figure 5)

309 Glycolysis is primarily via the pentose phosphate and the Entner-Doudoroff pathways in  
310 *Pseudomonas*.<sup>58</sup> Trehalose degradation to glucose is inferred in *P. putida* via the  
311 pathway trehalose degradation I, from its expected taxonomic range, in the MetaCyc  
312 database.<sup>59</sup> We observed a 1.3 fold increase in xylitol in exposed cells indicative of  
313 pentose interconversions.

314

315 Both trehalose and cellobiose were identified as significantly altered metabolites on  
316 exposure to more than one pharmaceutical: in *P. putida* exposed to propranolol,  
317 ibuprofen, acetaminophen and atenolol the concentration of trehalose was lowered  
318 significantly by 0.5, 0.4, 0.4, and 0.5; in *P. putida* exposed to propranolol and ibuprofen  
319 the concentration of cellobiose was also lowered significantly by 0.8 and 0.3,  
320 respectively. Trehalose has been observed to have a role as an osmoprotectant; for  
321 example trehalose concentration in *P. aeruginosa* was recently shown to be dependent  
322 on NaCl concentration<sup>60</sup>, and in response to solvent exposure in *Pseudomonas* sp.  
323 BCNU171.<sup>61</sup> In this study the concentration of both trehalose and cellobiose were  
324 reduced and we inferred from these changes that the disaccharides trehalose and  
325 cellobiose, which have a role as an energy reserve (both converted to D-glucose), are  
326 utilized as some energy consuming process(es) is (are) upregulated on exposure to  
327 propranolol. We confirmed that ATP concentrations were raised in *P. putida* exposed to  
328 propranolol (*vide infra* and Figure 6). Propranolol, which has a planar naphthalene  
329 structure and is known to interact with lipid membranes<sup>62</sup>, is present in the extracts of  
330 the exposed cells. It may be that an energy dependent efflux pump is one mechanism  
331 used to remove propranolol from the cell. *P. putida* KT2440 genome sequencing<sup>24</sup>  
332 revealed a large number of different efflux systems that may be involved in the active  
333 export of solvents, and the TolC outer membrane channel protein has been shown to be

334 upregulated in response to phenol.<sup>63</sup> Concurrent with solvent extrusion, a process with  
335 high energy demand partly required for the operation of efflux pumps, sugar uptake,  
336 glucose catabolic enzymes, and TCA cycle enzymes are induced.<sup>64</sup>

337

338 Amino acids were reduced in concentration on exposure to propranolol by 0.29 – 0.73  
339 fold (Table SI3 2 in the supplementary information.). By contrast, few significant  
340 alterations in amino acids were observed on exposure to other pharmaceuticals.  
341 Alterations were observed on exposure to ibuprofen (phenylalanine: 1.2 fold increase),  
342 diclofenac ( $\beta$ -alanine: 0.73 fold decrease) and acetaminophen (phenylalanine: 0.2 fold  
343 decrease, and glutamic acid: 0.72 fold decrease). Together with alterations in the protein  
344 complement of exposed cells observed in the FT-IR analysis this is consistent with *de*  
345 *novo* protein synthesis (possibly including an efflux system) in response to exposure.  
346 Using DNA array technology to investigate the response of *P. putida* KT2440 to  
347 toluene, o-xylene and 3-methylbenzoate Dominguez-Cuevas and co-workers observed  
348 major changes in genes related to amino acid biosynthesis and critical functions for  
349 protein production.<sup>65</sup> Strong induction of methionine biosynthesis was observed. In  
350 addition, leucine, isoleucine, tryptophan, serine and arginine biosynthesis was induced  
351 while catabolism of tryptophan and arginine was turned down, reflecting the need for  
352 amino acids in the new proteome found in cells exposed to toluene.

353

354 In *P. putida* exposed to propranolol we observed a significant 0.66 fold decrease in  
355 myoinositol which has a role in glycosylphosphatidylinositol (GPI)-anchor biosynthesis,  
356 and the anchoring of cell-surface proteins to the cell membrane.

357



358 In *P. putida* exposed to propranolol we observed that the concentrations of 2-  
359 monopalmitin and monostearin were lowered to near the analytical limit of detection  
360 and by 0.8, respectively. The level of octadecanoic acid was raised 8 fold, although the  
361 p-value from the ANOVA was marginally greater than the threshold p-value at  $2.14 \times$   
362  $10^{-2}$ . We observed a significant 2 fold increase in levels of glycerol-3-phosphate which  
363 has a major role in glycerolipid and glycerophospholipid metabolism, where it is the  
364 precursor to the phosphatidyl moiety and the two phosphatidyl residues linked by a  
365 glycerol moiety in cardiolipins. We observed a significant 0.49 fold decrease in level of  
366 heptadecanoic acid decreased, and a slight increase (1.15 fold) in the level of  
367 pentadecanoic acid. Other fatty acids detected were hexadecanoic, hexadecenoic, *cis*-9-  
368 octadecenoic acid, and octadecenoic acid methyl ester and were not altered significantly  
369 on exposure to propranolol.

370

371 In cells exposed to diclofenac, atenolol and mefenamic acid, the concentration of 9-  
372 octadecenoic acid methyl ester fell to the analytical limit of detection, and hexadecenoic  
373 acid lowered by 0.9 in cells exposed to ibuprofen and may be indicative of  
374 cyclopropane fatty acid formation. The unsaturated fatty acids *cis*-9-octadecenoic, *cis*-  
375 11-octadecenoic and *cis*-9-hexadecenoic acids are the precursors of the cyclopropane  
376 fatty acids found in *E. coli* and *P. putida*, *cis*-9,10- and *cis*-11,12-  
377 methyleneoctadecanoic (C<sub>19</sub>) and *cis*-9,10-methylenehexadecanoic (C<sub>17</sub>) acids <sup>66</sup>.  
378 However, methylation is to the esterified fatty acid in phospholipids (the C1 donor is S-  
379 adenoyslmethionine), and, since the extraction protocol for GC-MS did not allow  
380 detection of fatty acids from esterified lipids, we were unable to detect any  
381 corresponding alterations in C<sub>19</sub> or C<sub>17</sub> cyclopropane fatty acids. The concentration of  
382 pentadecanoic acid increased 1.1 fold in cells exposed to ibuprofen and acetaminophen.

383

384 Ramos and colleagues summarized several alterations in fatty acid composition in the  
385 bacterial response to solvent exposure<sup>52</sup>, which include *cis* to *trans* isomerization of  
386 esterified fatty acids, a shift in the ratio of saturated : unsaturated fatty acids and  
387 formation of C<sub>17</sub> cyclopropane fatty acids. Quantitative proteomics has revealed the  
388 upregulation of proteins involved in cell wall biosynthesis and plasma membrane fatty  
389 acids, and the outer membrane efflux protein TolC in the phenol-induced stress-  
390 response in KT2440.<sup>63</sup> The highest level of phenol-stimulation was observed for AccC-  
391 1. This is the enzyme encoding the first step of the fatty acid biosynthetic pathway and  
392 leads to an increase in the rate of fatty acid biosynthesis under phenol stress as a  
393 recovery mechanism for oxidatively damaged membrane phospholipids.

394 Propranolol is known to interact with lipid membranes and was observed in the  
395 methanol extracts of exposed cells. Tolerance to toluene in *P. putida* DOT-T1E has  
396 been suggested to be based on its exclusion by constitutive and inducible efflux pumps  
397 and rigidification of the cell membranes via phospholipid alterations.<sup>64</sup> A number of  
398 studies have looked at adaptive changes in membrane lipids in response to solvent  
399 exposure. Studies by Junker and Ramos showed that a major adaptive change observed  
400 in the solvent resistant strain *Pseudomonas putida* DOT T1E in response to solvent is  
401 *cis* to *trans* isomerization in membrane lipids, predominantly in  
402 phosphatidylethanolamines, which counteracts the increase in membrane fluidity caused  
403 by toluene.<sup>67</sup> The *cis:trans* ratio decreased from 7.5 to 1 when cells were grown in 1%  
404 toluene and changes were observed within 1 min of solvent exposure. The isomerase *cti*  
405 is located in periplasm where access to esterified phospholipids is possible and *cis* to  
406 *trans* isomerization is the main adaptive change in the short term, allowing cells to  
407 adapt immediately to environmental conditions in which a denser membrane packing is

408 a selective advantage. Cells gain time for *de novo* biosynthesis of membrane  
409 components as late as 15 min after solvent exposure. These changes include a shift in  
410 the ratio of saturated : unsaturated fatty acids and formation of C<sub>17</sub> cyclopropane fatty  
411 acids, synthesis of solvent extrusion pumps, modifications in lipid polysaccharides and  
412 alterations in membrane protein content.

413

414 *ANOVA analysis of ATP concentrations prior to and post exposure to propranolol:* We  
415 observed a significant rise in ATP concentration from 3.22 to 4.10 moles mg<sup>-1</sup> dry  
416 weight cells in *P. putida* after 1 h exposure to propranolol (Figure 6).

417

418 The critical p-value ( $\alpha$ ) here was assumed to be 0.01. p-values were calculated in an  
419 ANOVA analysis for the null hypothesis that the medians of the 2 groups are equal: the  
420 p-value for controls prior to exposure vs. controls 1 h after exposure =  $1.2 \times 10^{-1}$  and the  
421 p-value for exposed cells prior to exposure vs. 1 h after exposure =  $1.2 \times 10^{-5}$ , showing  
422 a significant difference at the  $\alpha = 0.01$  level in ATP concentration and energy demand in  
423 cells exposed to propranolol.

424

425 (Figure 6)

## 426 **Conclusions**

427 In conclusion, we have presented a novel metabolomics approach to investigate the  
428 effect of human pharmaceuticals on the environmentally relevant microorganism *P.*  
429 *putida* KT2440 (UWC1). Metabolic profiling using GC-MS coupled with univariate  
430 analysis and spring embedded correlation analysis was used to identify metabolites  
431 contributing to discrimination between cells exposed to the six drugs, and statistically

432 significant differences were observed for propranolol, diclofenac, ibuprofen and  
433 acetaminophen compared to untreated control cells.

434

435 The concentrations of several metabolites were altered significantly on exposure to a  
436 number of the pharmaceuticals and may be considered biomarkers of abiotic stress. The  
437 endogenous, metabolites 129 and 130 were significantly reduced in concentration in *P.*  
438 *putida* exposed to propranolol, and 129 was reduced in cells exposed to diclofenac and  
439 mefenamic acid. Concentrations of trehalose and metabolite 47 were also significantly  
440 reduced on exposure to propranolol, ibuprofen, acetaminophen and atenolol.

441

442 Six metabolites, 131, 134, 135, 139, 142, and 145, were raised in exposed cells from  
443 near or below the limit of detection in the control, and were not present in cells exposed  
444 to any other pharmaceutical in the study. Identification was not possible from the mass  
445 spectra which contained only low *m/z* ions. Further work using <sup>13</sup>C-labelled propranolol  
446 would determine whether propranolol is metabolized by KT2440, and whether these  
447 metabolites are endogenous metabolites or products of propranolol catabolism.

448

449 We also note that the growth conditions we have used (*viz.* R2A medium) are  
450 considerably more nutrient rich than what would normally be expected in an  
451 environmental water sample or found within benthic-sediment ecology (although in the  
452 benthos or fresh water sediment one would expect the APIs to be more concentrated).

453 Future work would be to investigate the use of the above markers of abiotic stress in a  
454 suitable ecosystem. Such an approach would involve target metabolite analysis  
455 encompassing significant sample clean up, specific metabolite extraction and targeted  
456 MS-MS for definitive metabolite identification and quantification.

457

458 With respect to exposure to propranolol, FT-IR analysis revealed changes in fatty acids  
459 and protein structure while GC-MS revealed alterations in energy reserves, amino acids  
460 and some fatty acids. Measurement of ATP concentrations in *P. putida* exposed to  
461 propranolol showed an increased level of ATP in exposed cells. These alterations are in  
462 agreement with previous studies which have shown that lipids in the membrane are  
463 altered to try to retain membrane integrity, and that energy dependent efflux pumps are  
464 used to remove toxic compounds from the cell. Additional studies undertaken by us  
465 include further investigation of the phospholipid and fatty acid alterations in *P. putida*  
466 exposed to propranolol, and these will be reported elsewhere.

467

468 We believe that this approach shows for the first time the value of developing a  
469 comprehensive metabolomics-based approach both for identifying discriminatory  
470 metabolites and their relationships to each other that reproducibly alter under abiotic  
471 stress. Moreover, this approach allows the investigation of mechanisms of response to  
472 these stresses in environmentally relevant microbes and future work will investigate  
473 these effects in complex microbial communities.

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478

#### 479 **Conflict of Interests**

480 All authors have no conflict of interest to declare.

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484 Supplementary information is available online

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## 633 Figure Legends

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635

### Figure 1.

636

#### a) Cross-validated PC-CVA models for the FT-IR spectra of *P. putida* exposed to the six pharmaceuticals.

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The FT-IR spectra were normalized and variables scaled to unit variance. 26 PCs (99.99% explained variance) and 6 CVs were used in the analysis trained on 4 class replicates (filled triangles). Test data (a fifth, unknown, class replicate) are marked with an asterisk (open triangles). Key; acetaminophen red, atenolol gold, diclofenac green, ibuprofen cyan, mefenamic acid blue, propranolol purple, control grey. Circles represent the 95% confidence limit from the group centres here constructed around each group mean by the  $\chi^2$  distribution on two degrees of freedom. Cells exposed to propranolol and ibuprofen are separated along PC-CV1; those exposed to mefenamic acid are separated along PC-CV2.

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#### b) Examination of the loadings for PC-CV1 from the PC-CV analysis.

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Significant loadings with amplitude  $>2SD$  from the mean are shown in blue overlaid with the FT-IR spectrum for propranolol (green) and the FT-IR spectrum for the cells exposed to propranolol at  $50\mu\text{g mL}^{-1}$  (blue). Several high loadings for PC-CV1 occur at significant wavenumbers for propranolol, the region corresponding to aliphatic C-H, and hence bacterial fatty acids, at  $2919$  and  $2850\text{cm}^{-1}$ , and the regions corresponding to the amide I bands in protein structures at  $1655\text{cm}^{-1}$  ( $\alpha$ -helical structures),  $1709$ ,  $1659$  and  $1630\text{cm}^{-1}$  ( $\beta$ -sheet structures).

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### Figure 2. PC-CVA model rebuilt using only absorbances at wavenumbers significant for bacterial fatty acids.

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The model used 12 PC scores (99.9% explained variance) and shows separation of *P. putida* exposed to propranolol (purple) from control cells (grey). Other exposures were not separated from the control, revealing that observed lipid alterations are specific to exposure to propranolol.

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### Figure 3. Altered metabolites in *P. putida* exposed to propranolol.

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As a visual method for assessing significance of metabolites as discriminating biomarkers the area under the ROC curve (AUC) is plotted versus p-value from the ANOVA analysis. If a metabolite has an AUC = 0.5 it is equally distributed between the two classes. A metabolite with an AUC = 1 is diagnostic of the class. Brown open circles denote fold increases, and grey open circles fold decreases, in metabolite concentration in exposed cells. Metabolites with an AUC  $> 0.7$  and a p-value  $< 1.77 \times 10^{-2}$  (the FWER threshold for a critical p-value equivalent to 0.05) are labeled. Some labels have been moved for clarity. Metabolites with an AUC  $> 0.85$  and a p-value  $< 1.77 \times 10^{-2}$  were considered significant and selected for correlation analysis.

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Figure 4. Spring embedded correlation plots showing correlation between 43 metabolites a) in cells exposed to water and b) in cells exposed to propranolol. Propranolol is correlated with energy-related metabolites: a sugar phosphate, trehalose and cellobiose; correlation is extended through metabolite 139, one of 3 metabolites with the lowest p-value whose concentration was raised from near the limit of detection, to succinic acid, a metabolite of the TCA cycle. In cells exposed to water trehalose and cellobiose are not correlated with succinic acid. Propranolol is also correlated with glycerol-3-phosphate, a precursor to the phosphatidyl group in glycerophospholipids.

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Figure 5. Alterations in the concentration of energy related metabolites in *P. putida* exposed to propranolol identified from the ANOVA analysis. Trehalose and cellobiose, which have a role as energy reserves, were reduced in concentration in exposed cells, while succinic acid and sugar phosphates (increased in concentration in exposed cells).

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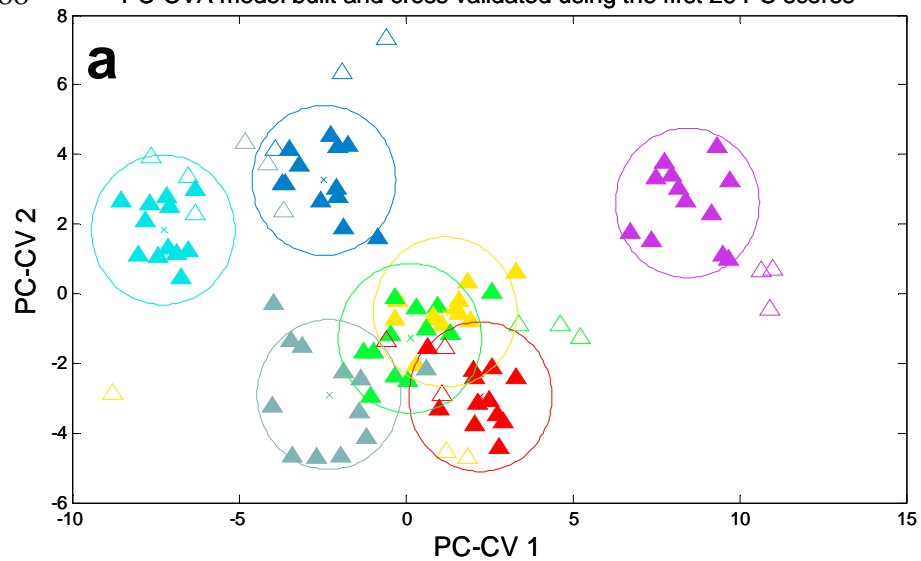
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Figure 6. Alterations in ATP concentration in *P. putida* exposed to propranolol. The median ATP concentration prior to and 1h after exposure in cells exposed to water as a control and cells exposed to propranolol. p-values were calculated in an ANOVA analysis for the null hypothesis that the medians of the 2 groups are equal: the p-value for controls prior to exposure vs. controls 1 h after exposure =  $1.2 \times 10^{-1}$  and the p-value for exposed cells prior to exposure vs. 1 h after exposure =  $1.2 \times 10^{-5}$ , showing a significant difference in ATP concentration and energy demand in cells exposed to propranolol.

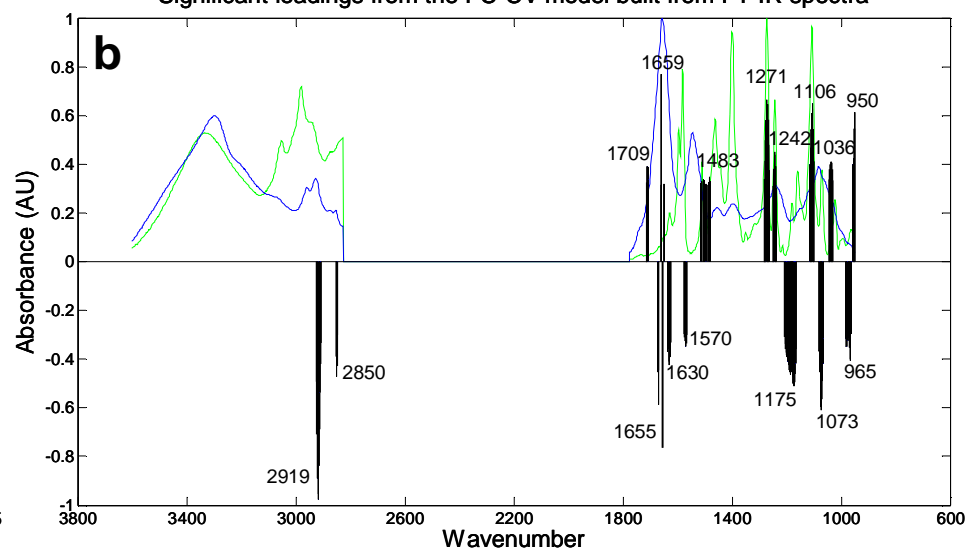
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687 Figure 1

688 PC-CVA model built and cross validated using the first 26 PC scores



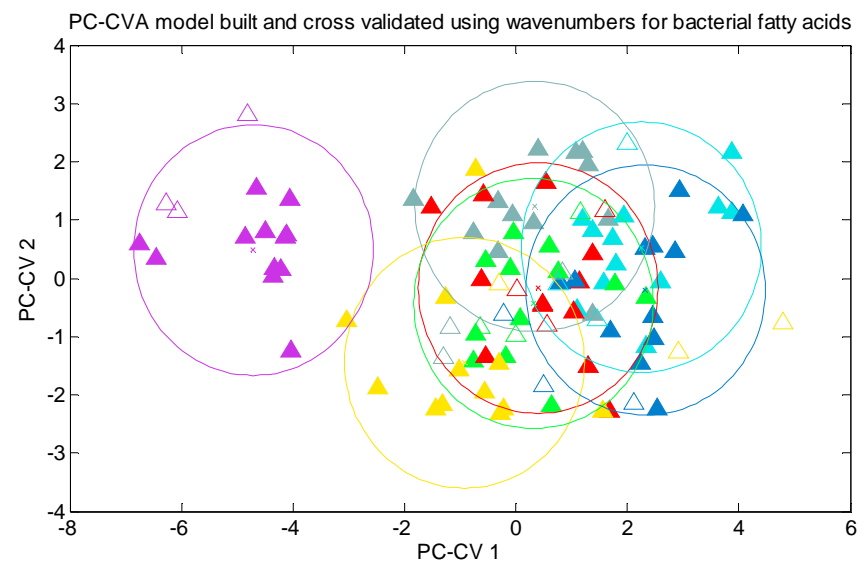
Significant loadings from the PC-CV model built from FT-IR spectra



689

Figure 2

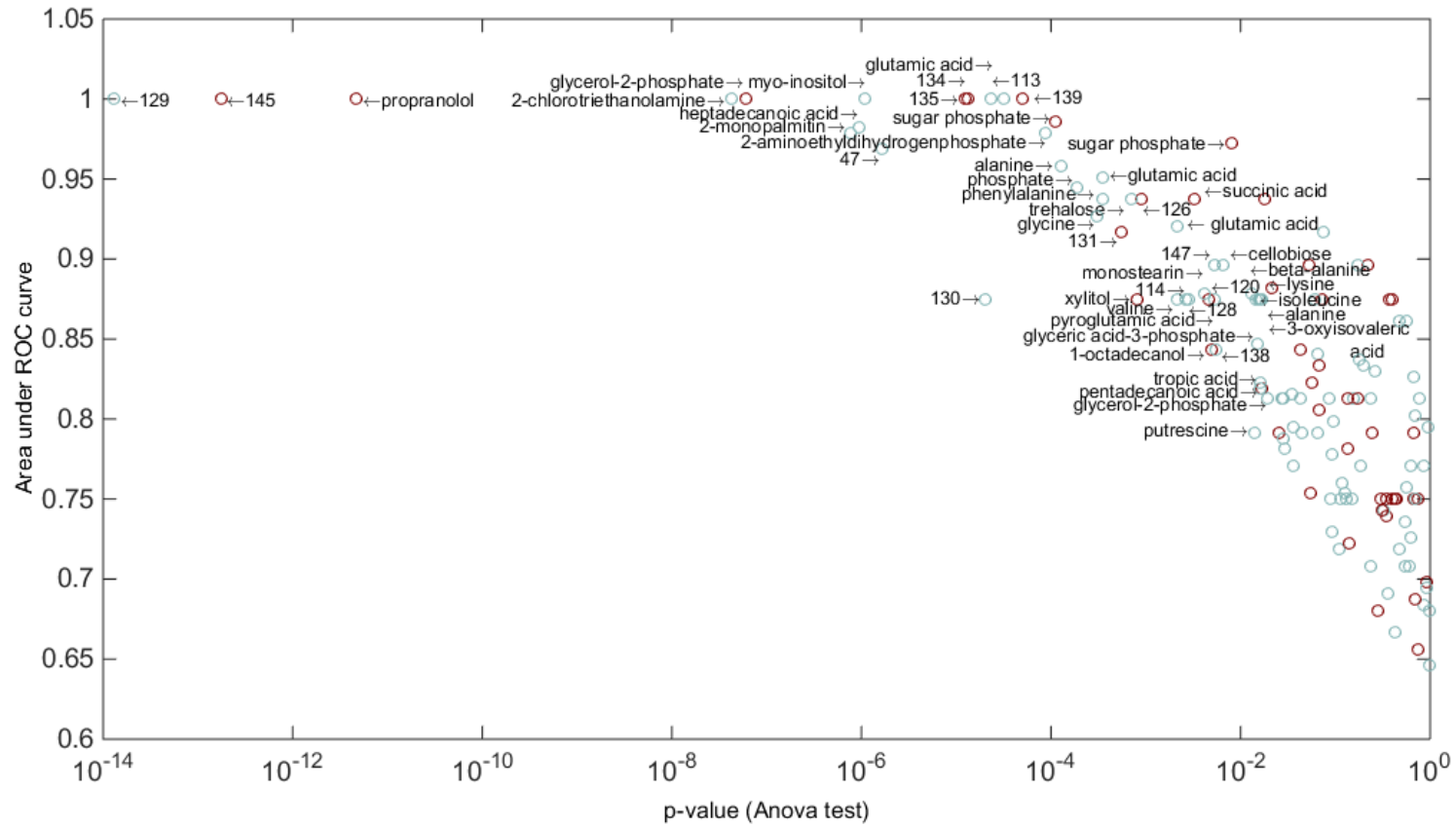
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Figure 3

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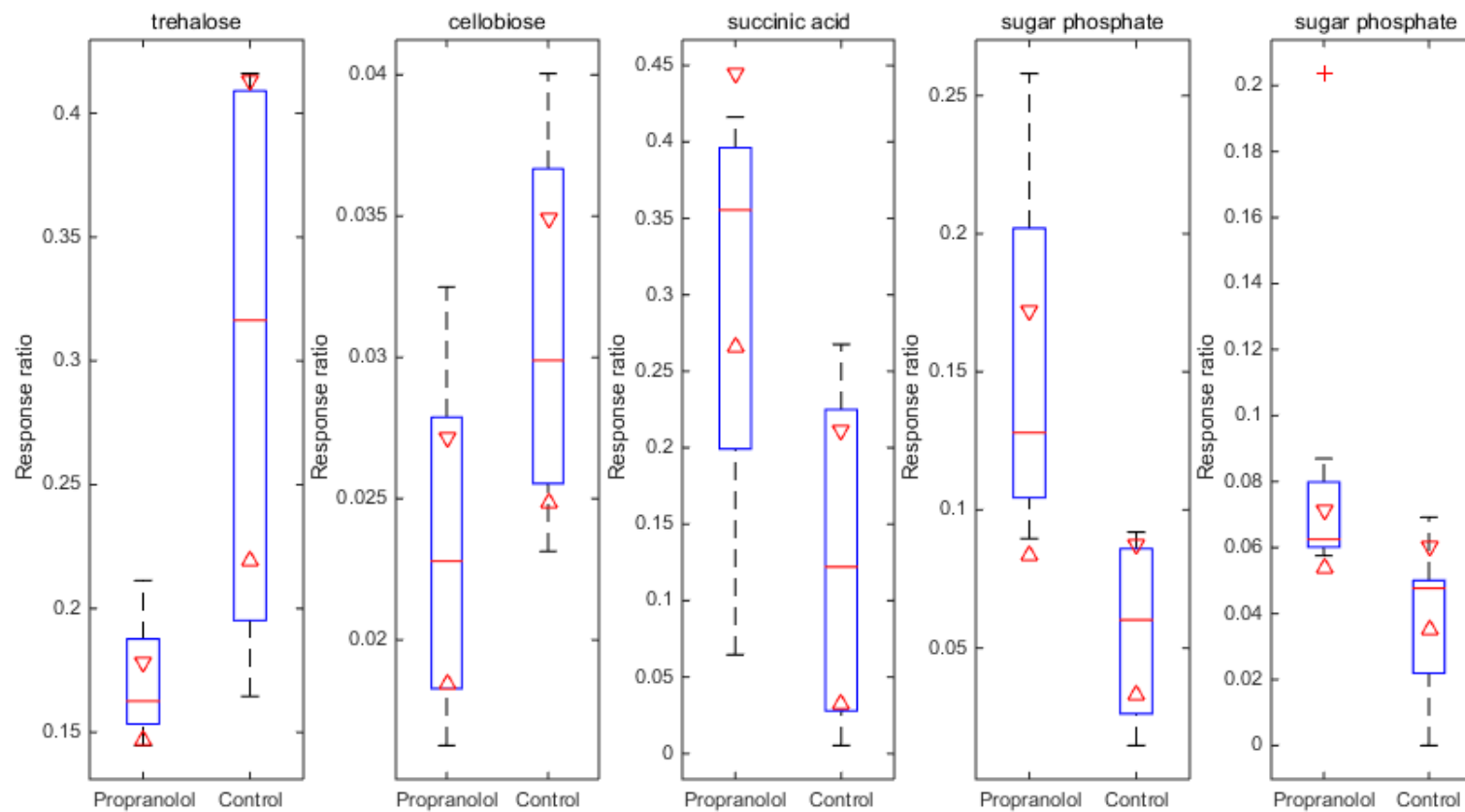


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694 Figure 4  
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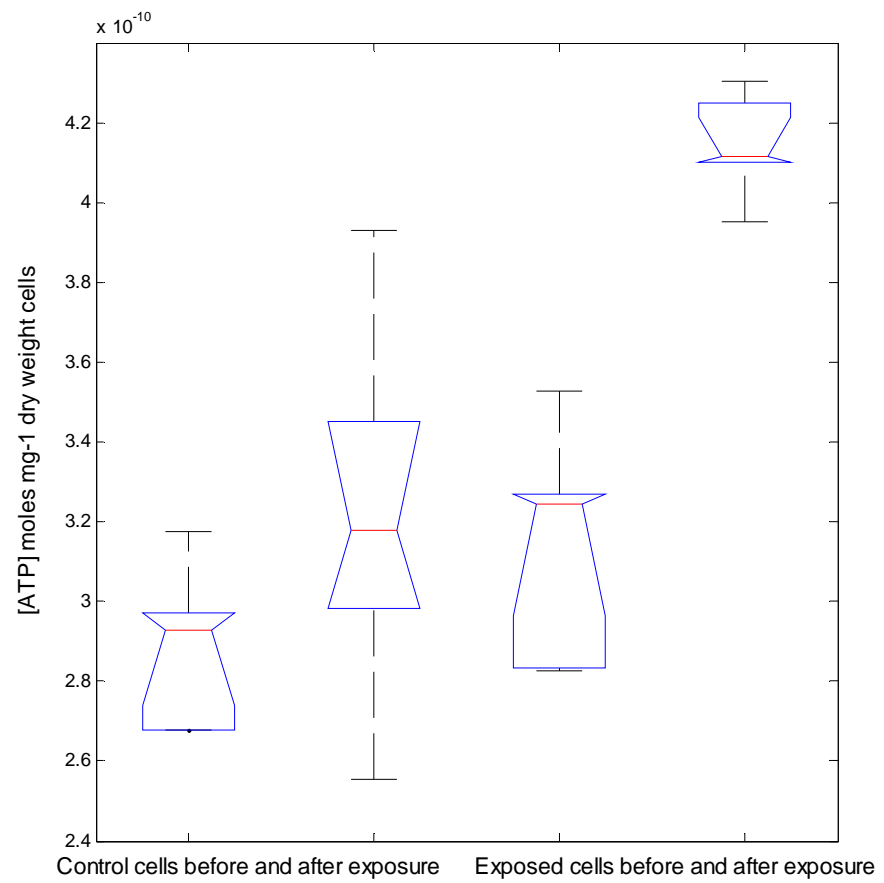


697 Figure 5



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700 Figure 6



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