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

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

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Abstract

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

Introduction

Studies spanning the last 20 years, have shown that human pharmaceuticals are present in waste water treatment plants (WWTPs), rivers and estuaries at the ng L^{-1} or low µg L^{-1} level throughout Europe and in the United States.[1-9] In monitoring studies in the UK 13 pharmaceuticals selected from priority lists of the UK Environment Agency and the Oslo and Paris Commission (OSPAR) were detected at concentrations ranging from 11 – 69,570 ng L^{-1} in raw WWTP effluent [1].

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

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

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

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

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

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

laboratory conditions, where those conditions specifically serve to mimic scenarios encountered in the natural environment.[38] There is considerable potential for omic profiling methods to progress significant advances in regulatory ecotoxicology, with applications including biomarker development and risk assessment for toxicant exposure, and evaluation of metabolic responses to environmental stressors.[39, 40] Reviews of recent applications in environmental metabolomics can be found in [41, 42].

In whole organism fingerprinting FT-IR spectroscopy measures bond vibrations of functional chemical groups in cell constituents such as DNA and RNA, proteins, lipids and carbohydrates. FT-IR may be used as a screening tool providing rapid discrimination between samples, through measurement of overall phenotypic changes in a sample without specific identification of the individual metabolites responsible. In contrast, metabolic profiling by GC-MS provides semi-quantification and, where possible, the definitive identification of metabolites through retention time and mass spectrum matching. Subsequent data analysis can then reveal discriminatory metabolites.[33]

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

ng L^{-1}, mefenamic acid 234-959 ng L^{-1} and propranolol 83-291 ng L^{-1}. Roberts and Thomas suggest that it would also be beneficial to determine levels of parent compounds present in sewage sludge, in order to determine levels of adsorption.[1]

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

Materials and Methods

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

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

Results and Discussion

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

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

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

more rigid cell membrane structure, and synthesis of drug efflux pumps in order to remove toxic substances from the cell.[52]

The PC-CVA was repeated using absorbances at wavenumbers selected from those with significantly high loadings shown in Figure 1b. Rebuilding the model with absorbances only at wavenumbers significant for bacterial fatty acids showed discrimination between *P. putida* exposed to propranolol and the control, and no discrimination of any other exposure from the control (Figure 2). Thus, observed lipid alterations were specific for exposure to propranolol. PC-CVA models built using absorbances at selected wavenumbers significant for the fingerprint region showed little difference from the model using the entire dataset, affording no new information, while there were too few wavenumbers significant for protein to use successfully in a PC-CVA model.

(Figure 1)

(Figure 2)

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

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

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

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

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

(Figure 3)

(Figure 4)

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

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

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

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

(Figure 5)

309 Glycolysis is primarily via the pentose phosphate and the Entner-Doudoroff pathways in
310 *Pseudomonas.*[58] 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.[59] 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[60], and in response to solvent exposure in *Pseudomonas* sp.
323 BCNU171.[61] 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 utitilized 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[62], 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[24]
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

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

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

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

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

In cells exposed to diclofenac, atenolol and mefenamic acid, the concentration of 9-octadecenoic acid methyl ester fell to the analytical limit of detection, and hexadecenoic acid lowered by 0.9 in cells exposed to ibuprofen and may be indicative of cyclopropane fatty acid formation. The unsaturated fatty acids *cis*-9-octadecenoic, *cis*-11-octadecenoic and *cis*-9-hexadecenoic acids are the precursors of the cyclopropane fatty acids found in *E. coli* and *P. putida*, *cis*-9,10- and *cis*-11,12-methyleneoctadecanoic (C_{19}) and *cis*-9,10-methylenehexadecanoic (C_{17}) acids [66]. However, methylation is to the esterified fatty acid in phospholipids (the C1 donor is S-adenoyslmethionine), and, since the extraction protocol for GC-MS did not allow detection of fatty acids from esterified lipids, we were unable to detect any corresponding alterations in C_{19} or C_{17} cyclopropane fatty acids. The concentration of pentadecanoic acid increased 1.1 fold in cells exposed to ibuprofen and acetaminophen.

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

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

a selective advantage. Cells gain time for *de novo* biosynthesis of membrane components as late as 15 min after solvent exposure. These changes include a shift in the ratio of saturated : unsaturated fatty acids and formation of C_{17} cyclopropane fatty acids, synthesis of solvent extrusion pumps, modifications in lipid polysaccharides and alterations in membrane protein content.

ANOVA analysis of ATP concentrations prior to and post exposure to propranolol: We observed a significant rise in ATP concentration from 3.22 to 4.10 moles mg^{-1} dry weight cells in *P. putida* after 1 h exposure to propranolol (Figure 6).

The critical p-value (α) here was assumed to be 0.01. 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 = 1.2×10^{-1} and the p- value for exposed cells prior to exposure *vs.* 1 h after exposure = 1.2×10^{-5}, showing a significant difference at the $\alpha = 0.01$ level in ATP concentration and energy demand in cells exposed to propranolol.

(Figure 6)

Conclusions

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

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

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

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

We also note that the growth conditions we have used (*viz*. R2A medium) are considerably more nutrient rich that what would normally be expected in an environmental water sample or found within benthic-sediment ecology (although in the benthos or fresh water sediment one would expect the APIs to be more concentrated). Future work would be to investigate the use of the above markers of abiotic stress in a suitable ecosystem. Such an approach would involve target metabolite analysis encompassing significant sample clean up, specific metabolite extraction and targeted MS-MS for definitive metabolite identification and quantification.

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

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

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Conflict of Interests

All authors have no conflict of interest to declare.

Supplementary information is available online

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Figure Legends

Figure 1.

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

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 χ^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.

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

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 50µg 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 2850cm^{-1}, and the regions corresponding to the amide I bands in protein structures at 1655cm^{-1} (α-helical structures), 1709, 1659 and 1630cm^{-1} (β-sheet structures).

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

Figure 3. Altered metabolites in *P. putida* exposed to propranolol. 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 < 1.77×10^{-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 < 1.77×10^{-2} were considered significant and selected for correlation analysis.

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.

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**.**

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 =1.2×10^{-1} and the p- value for exposed cells prior to exposure *vs.* 1 h after exposure = 1.2×10^{-5}, showing a significant difference in ATP concentration and energy demand in cells exposed to propranolol.

687 Figure 1

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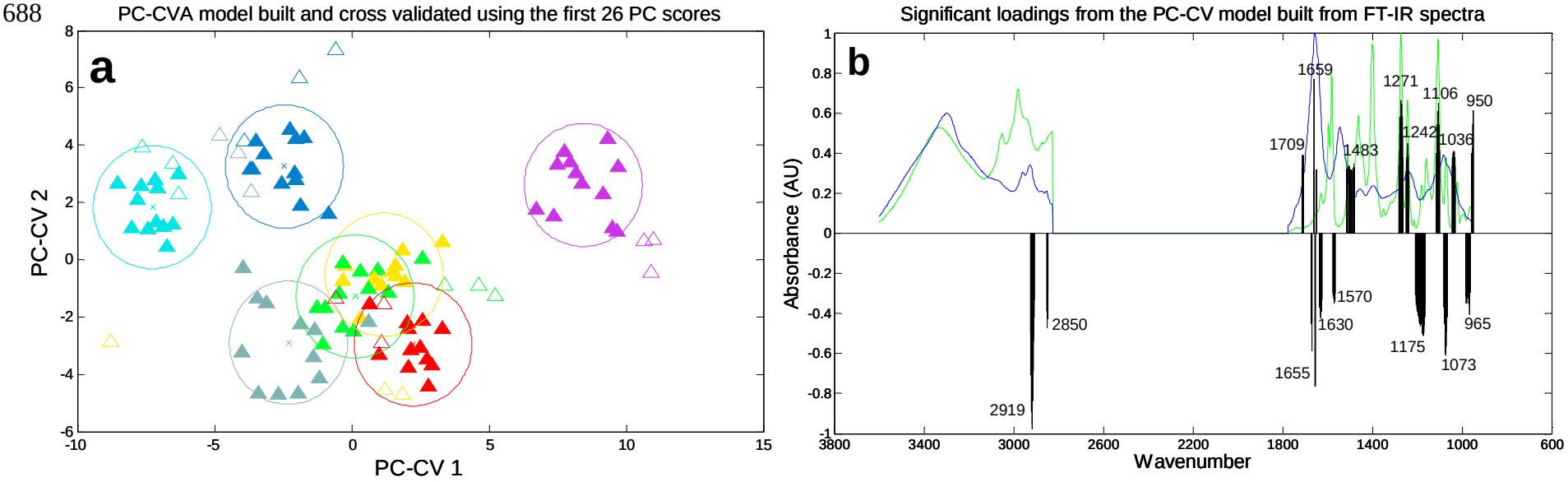

689 Figure 2

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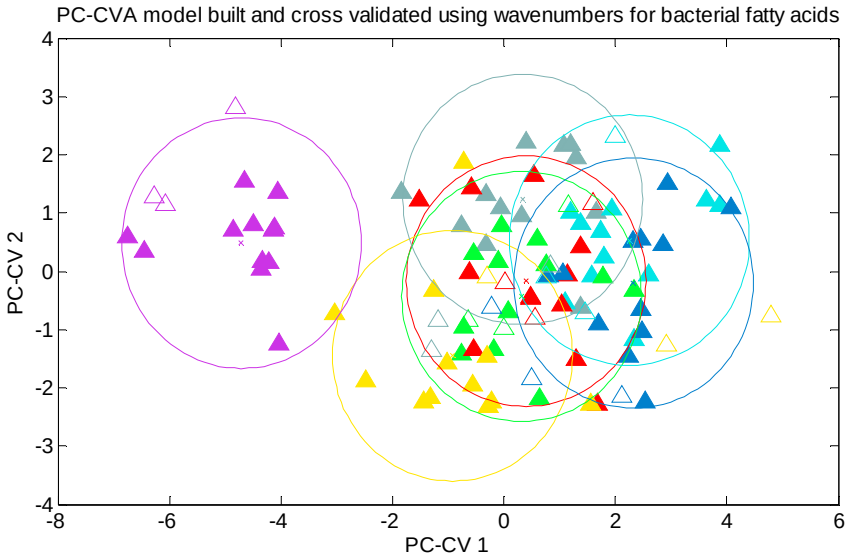

691 Figure 3

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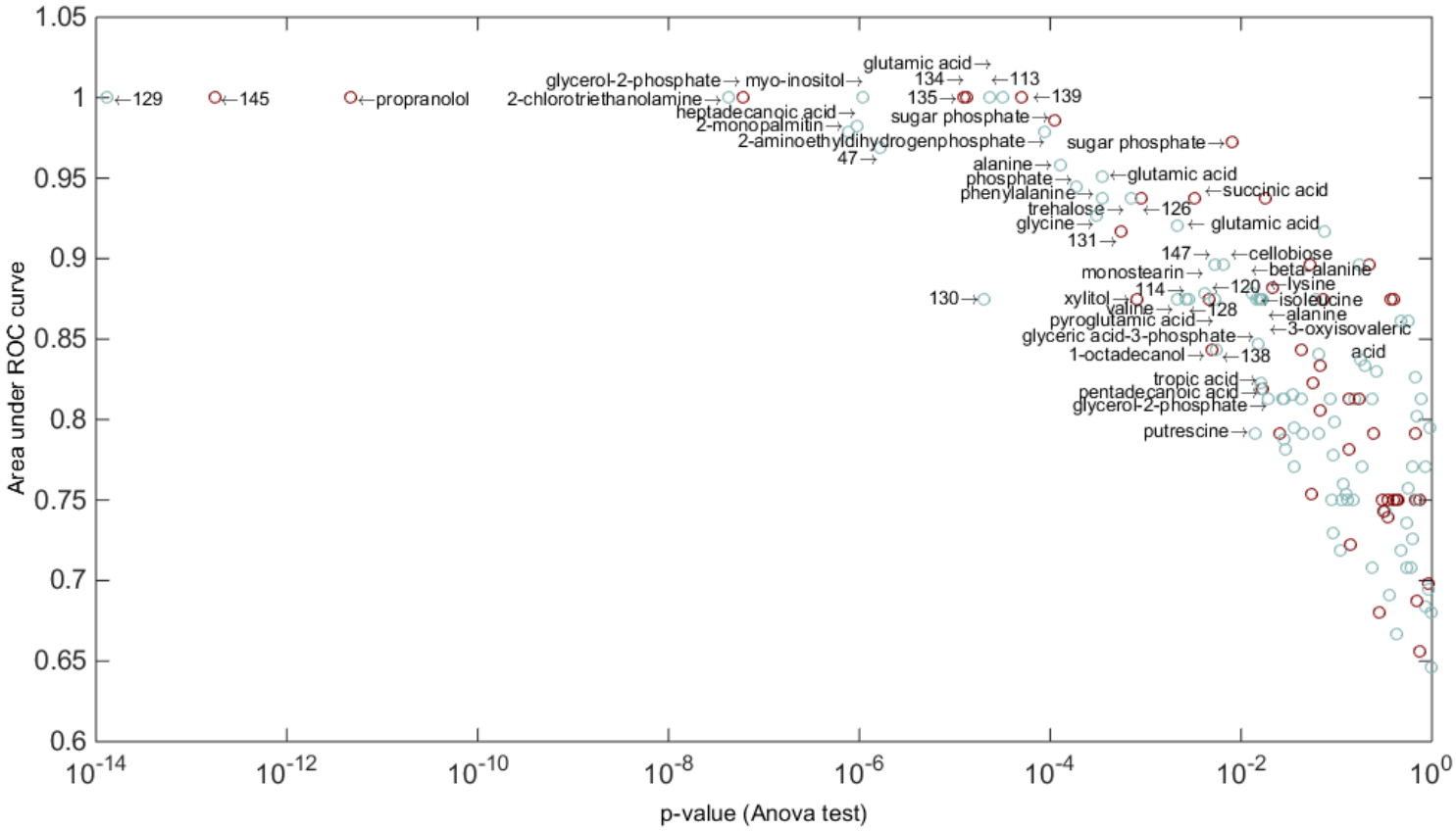

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694 Figure 4
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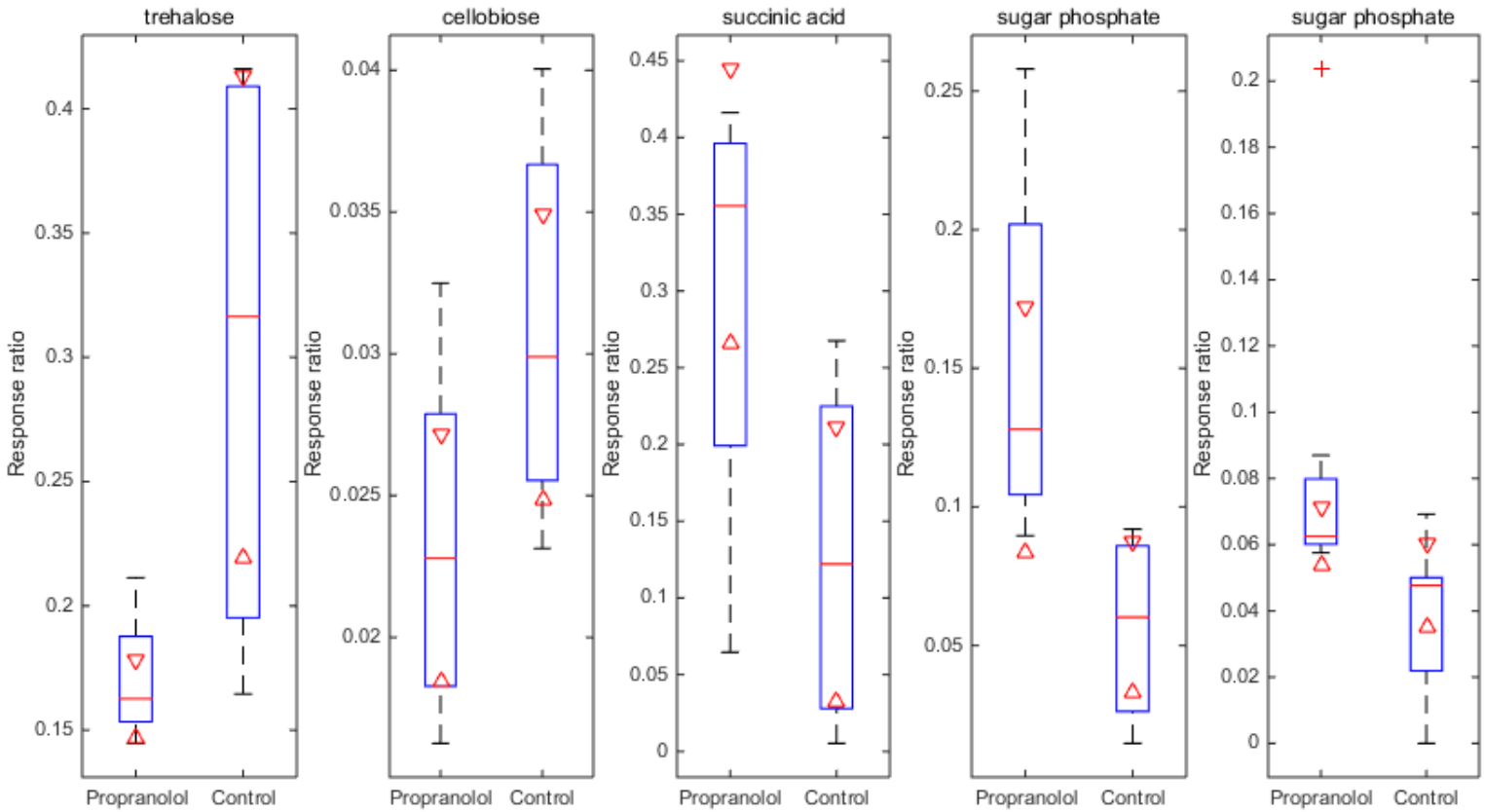
697 Figure 5



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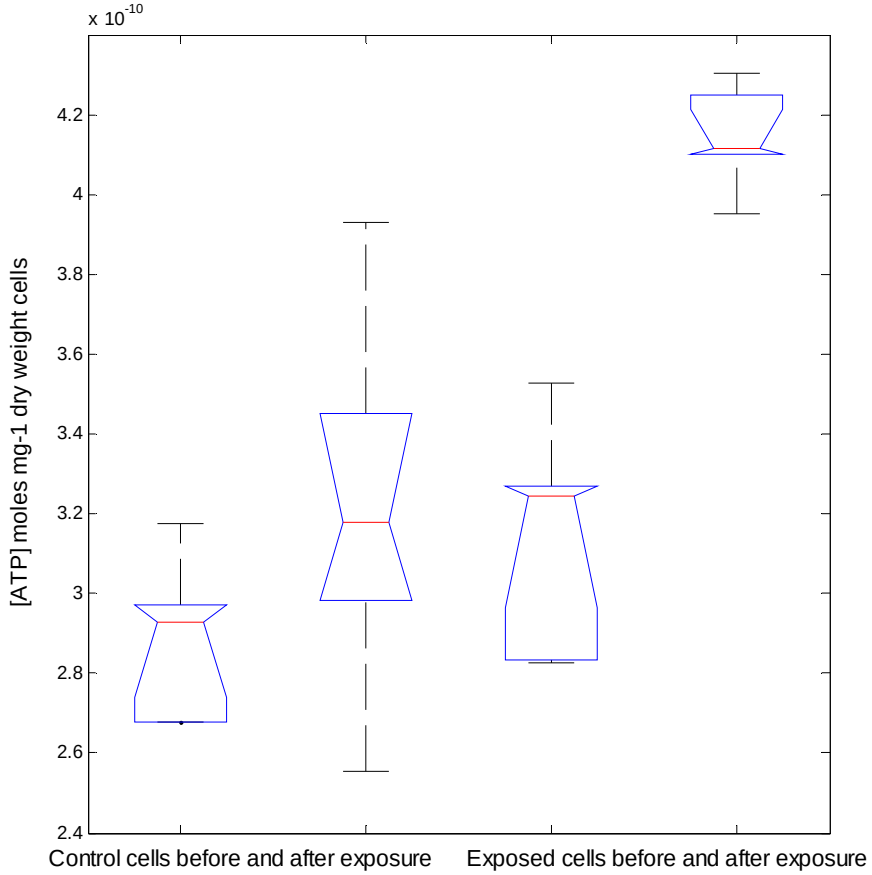
700 Figure 6



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