A Multidimensional approach to the study of the lipidome and metabolome of the green Algae *Chlamydomonas reinhardtii*

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Introduction

- Active pharmaceutical ingredients (APIs) and their metabolites are a class of emerging environmental pollutants that are commonly being discharged from wastewater treatment works effluents [1].
- Bioaccumulation of drug residues in aquatic organisms is not fully characterised, however several cases to date have demonstrated the serious impact that such compounds have on the whole ecosystem from aquatic microorganisms to wildlife.
- The β-blocker propranolol (figure 1) is a chiral pharmaceutical compound that exists as two enantiomers (labeled (R) or (S)). Approximately 56% of APIs currently employed are chiral and approximately 90% of these are administered therapeutically as the racemate.
- *Chlamydomonas reinhardtii* is a model algae employed to study diverse biological processes. When stressed under controlled conditions, perturbations in the lipidome have previously been reported [2]. Here *C. reinhardtii* was treated with three different concentrations of (R) and (S) propranolol and pertubations within the cell were investigated using Fourier Transform Infrared (FT-IR) Spectroscopy and Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS).

Experimental

- 1132C wild type *C. reinhardtii* cells were grown in 20 mL culture tubes in triplicate on an orbital shaker at 120 rpm at 22 ºC with a 16-h light:8-h dark light regime and a photon flux of approximately 150 µmol m^{-2} s^{-1} for 7 days.
- Standard tris-acetate-phosphate (TAP) medium was used for the control system and spiked with concentrations of 1, 30 and 150 µg/mL (R)- and (S)-propranolol hydrochloride (mw 295.8) to establish controlled stressed growth conditions.

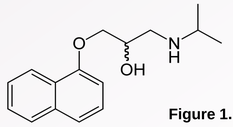


Figure 1. Structure of propranolol

- At day 7, UV absorbance at 680 nm was recorded for each cell culture to gauge the optical density of chlorophyll containing cells. This is a good indication of cell viability and an insight into how the cultures grew.
- To quench cell metabolism the cultures were then spun down, rapidly washed and frozen in liquid nitrogen. Cells were re-suspended in water prior to analysis.

Analysis of algae cells by FT-IR Spectroscopy

- Samples were spotted in triplicate onto a prepared ninety-six-well silicon plate and triplicate spectra obtained using different positions in each well; a total of nine spectra (technical replicates) per sample were collected. Spectra were collected with an Equinox 55 FT-IR spectrometer (Bruker Optics Ltd.) in transmission mode using a deuterated triglycine sulfate detector over a wavelength range from 4000 to 600 cm^{-1} and with a resolution of 4 cm^{-1}.

Analysis of algae cells by ToF-SIMS

- Samples were spotted onto prepared silicon wafers and dried in a vacuum desiccator for 24 hours.
- ToF-SIMS analysis was performed on a BioToF-SIMS instrument previously described by Braun *et al* [3]. The primary ion beam used for analysis was 20 keV Au_3^+. A primary ion fluence of 2×10^{11} ions cm^{-2} was used for each analysis.

[1] J.L. Zhou *et al,* Journal of Hazardous Materials. (2009), 166, 655
[2] T. Cakmak *et al.,* Biotech. and Bioeng. (2012), 109(8), 1947
[3] R.M. Braun *et al.,* Rapid Commun. Mass Spectrom. (1998), 12(18), 1246

Results and Discussion

Optical Density of *C. reinhardtii* post-treatment with (R)- and (S)-propranolol

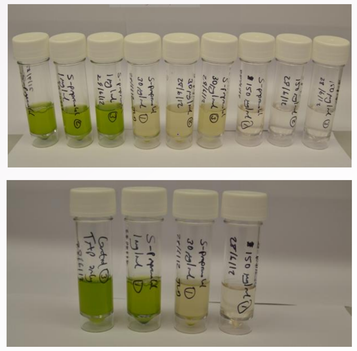
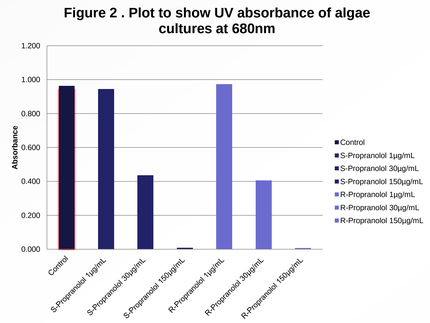


Figure 2 . Plot to show UV absorbance of algae cultures at 680nm



- Results in figure 2 show a clear concentration effect in cell viability and optical density with the varying concentrations of (R)- and (S)-propranolol when compared with the control. Cell viability was very poor at 150 µg/mL (R)- and (S)-propranolol therefore yielding little biomass which excluded this concentration from subsequent data analysis.

Infrared Spectroscopy of *C. reinhardtii*

- Figure 3 shows baseline corrected and averaged spectra for the *C. reinhardtii* cultures under different propranolol treatments.

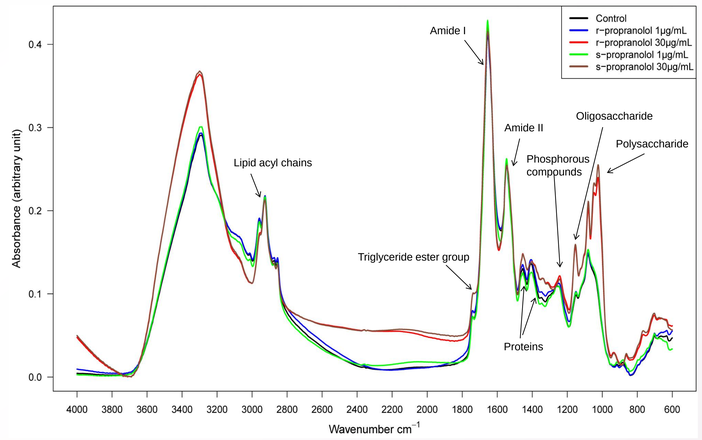


Figure 3. Representative processed FT-IR spectra for *C. reinhardtii* exposed to (R)- and (S)-propranolol at 1 µg/mL and 30 µg/mL . Control samples which were not exposed to propranolol are included.

- All components of the *C. reinhardtii* cells such as amino acids, lipids and carbohydrates contribute to the FT-IR spectra.
- IR absorbance bands can be attributed to vibrations as described by Cakmak *et al* [2]. Significant concentration effects can be observed between the 1 µg/mL and 30 µg/mL propranolol concentrations which are further confirmed in the loadings plots shown in figure 4.
- Observable chemical differences due to species such as oligosaccharides and polysaccharides shall be investigated further by LC/MS.

Infrared Spectroscopy Multivariate Data Analysis

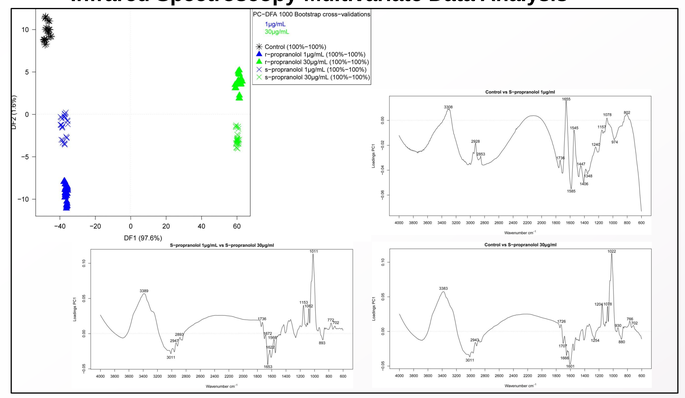


Figure 4. PC-DFA and PCA loadings plot showing the differences between the spectra of control *C. reinhardtii* and algae treated with 1 and 30 µg/mL (S)- propranolol

- The PC-DFA plot shows that the main contribution to the group separation between cells is that of concentration of propranolol, which explains 97.6% of the data variance. Cells treated with 1 µg/mL propranolol sit very close to the control cells in DF1 but are clearly separated in DF2, however the 30 µg/mL sample is separated in both DF1 and DF2.
- The enantiomeric effects observed which separate (R) and (S)-propranolol groups in DF2 are of particular interest and require further investigation.

ToF-SIMS analysis of *C. reinhardtii*

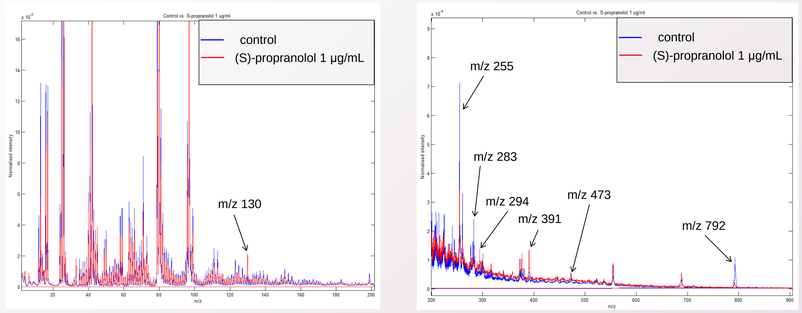


Figure 5. Negative ion characteristic ToF-SIMS spectra for the *C. reinhardtii* contol overlayed with the 1 µg/mL (S)-propranolol algae cells.

- The preliminary negative ion ToF-SIMS data (figure 5) shows clear differences in both presence/absence of peaks and peak abundance indicating that the effects of propranolol as observed by IR can be further characterised by ToF-SIMS.

Conclusion and Future Work

- The growth data clearly shows that both enantiomers of propranolol have a biological effect on *C. reinhardtii.* At higher concentrations growth was retarded therefore the next controlled experiment will titrate at concentrations between 1 µg/mL and 30 µg/mL propranolol.
- Preliminary ToF-SIMS data looks promising and future work will produce a larger data set which can be statistically analysed and assignments to significantly different peaks will be proposed.
- Raman spectroscopy and LC/MS will be employed to provide complementary data to contribute to the biochemical basis for classification.