Application Note

Environmental

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Assessing the Mitochondrial Toxicity of Environmental Pollutants Using the Agilent Seahorse XF Extracellular Flux Analyzer

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Abstract

This application note describes an *in vitro* bioassay workflow that uses the Agilent Seahorse XF Extracellular Flux Analyzer to measure the impact of surface water samples and individual organic chemicals, including pesticides known as mitochondrial toxicants, on metabolic function. Three experimental designs were proposed to detect and quantify the effect of samples (chemicals or water extracts) on the oxygen consumption rates (OCR) of HepG2 cells to differentiate three mechanisms of mitochondrial toxicity: ATP synthase inhibition, electron transport chain disruption, and proton gradient uncoupling. The resulting OCR time profiles were used to develop concentration-response curves for all three endpoints and derive effect concentrations that trigger 10% of the maximum effect caused by a potent ATP synthase inhibitor, electron transport chain inhibitor, or uncoupler. The applicability of the workflow was evaluated for environmental contaminants with known mechanisms of mitochondrial toxicity: the herbicide bromoxynil, the fungicides azoxystrobin and pyraclostrobin, and the biocide tributyltin. The workflow confirmed the predominant modes of toxicity for the contaminants tested. Following assay validation, river water samples downstream from a wastewater treatment plant were also tested. The workflow was able to reveal mitochondrial inhibition due to uncoupling and electron transport chain inhibition in real surface water samples.

Introduction

Limited information exists about exposure and toxicity for tens of thousands of chemicals in regular use. In vitro bioassays can enhance understanding of the biological activity of chemicals in environmental samples and their potential environmental impacts, while prioritizing chemicals for potential regulation. In addition, *in vitro* assays are increasingly used in toxicology testing to supplement multiple- and whole-organism testing. In vitro assays require less time and money than traditional testing, and thus offer substantial benefits for risk assessment studies and routine environmental monitoring.

Mitochondria play an important role in the cellular physiology of eukaryotic organisms and are responsible for most cellular energy production. Environmental pollutants are known to disturb energy production in mitochondria in three ways: (1) by interfering with adenosine triphosphate (ATP) synthase; (2) by uncoupling the inner and outer mitochondrial membranes, which interferes with the proton gradient required for ATP production; and (3) by inhibiting the protein complexes that create the electron transport chain's electrochemical gradient (Figure 1).

This application note describes an *in vitro* assay workflow that uses the Agilent Seahorse XF Extracellular Flux Analyzer to identify and measure the links between various chemicals and their biological impact on metabolic function, using the example of HepG2 cells. The method allows the differentiation between the three modes of action (MOA) of mitochondrial toxicity outlined in Figure 1.

The Agilent Seahorse XFe96 Analyzer is designed to measure oxygen consumption rate (OCR)—a key indicator of mitochondrial respiration, glycolysis, and ATP production rate—in live cells, in a 96-well plate format. XF Analyzers perform compound addition and mixing, label-free analytical detection, and automatic measurement of OCR in real time. Further details describing the methodology presented in this application note are available in the complementary publication *Mitochondrial Toxicity of Selected Micropollutants, Their Mixtures, and Surface Water Samples Measured by the Oxygen Consumption Rate in Cells*.[1] The use of a cellular assay to determine the mitochondrial impact of chemical exposure has been validated by the Irish EPA (in conjunction with Luxcel Biosciences, now a part of Agilent).[2] Similarly, the nontargeted analysis approach to chemical risk assessment has been validated by the US EPA using the high-resolution liquid chromatography/quadrupole TOF (LC/Q-TOF) technique with a Seahorse XF-based mitochondrial toxicity assay.[3]

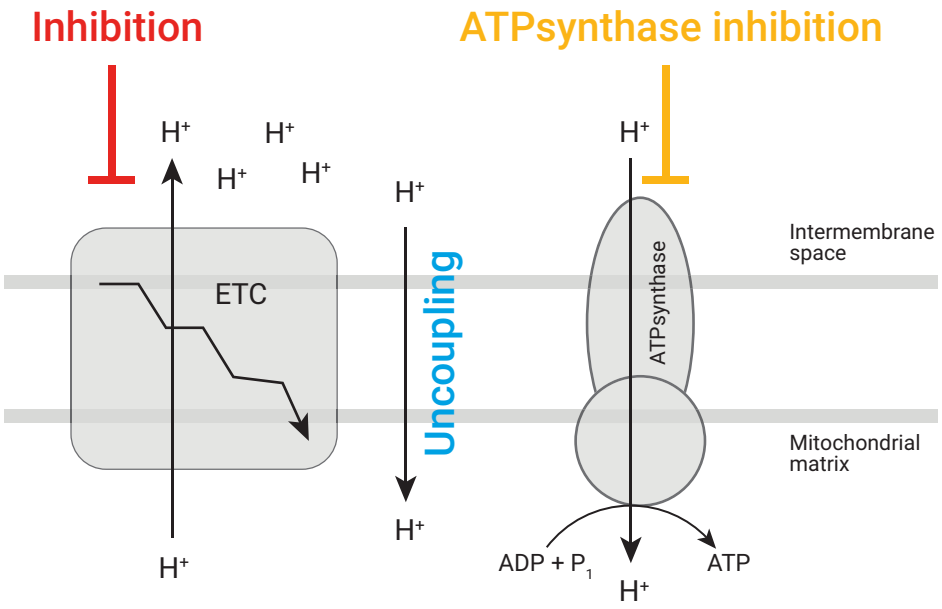

Figure 1. Mitochondrial energy production disruption mechanisms caused by chemical exposure include interference with adenosine triphosphate (ATP) synthase, uncoupling of the inner and outer mitochondrial membranes, which interferes with the proton gradient required for ATP production, and inhibition of the protein complexes that create the electron transport chain's (ETC) electrochemical gradient.

Common approach

All experiments were divided into four time segments, depicted with the positive controls in Figure 2. In each experiment, the basal respiration was measured for three measurement cycles during Segment 1. One measurement cycle consisted of 3 minutes measuring the cellular OCR and three minutes of mixing by lifting the sensors up and down. Then oligomycin, an inhibitor of ATP synthase, was added and the OCR was measured for nine cycles during Segment 2, corresponding to an incubation time of 54 minutes. The longer incubation time was used to increase assay robustness for ATP Synthase inhibitors and assure sufficient time for cellular uptake kinetics. At the beginning of Segment 3, the uncoupler 2,4-dinitrophenol (2,4-DNP) was added at 80 µM (causing 95% of maximum OCR), and the OCR was measured for three cycles. At the start of Segment 4, rotenone and antimycin A (Rot/AA) were added at 0.5 µM, completely inhibiting the electron transport chain, and the OCR was measured for another three cycles.

For each of the workflows, one of the reference chemicals (oligomycin, 2,4-DNP, or Rot/AA) was replaced by the chemical under investigation or the environmental sample to check if the chemical under investigation or the environmental sample activated the respective specific mode of action. In general, every endpoint was measured over three cycles but the endpoint under investigation used nine cycles of three plus 3 minutes. Positive control, negative control, and sample were tested on the same 96-well plate in different dilutions. For simplicity, all figures here show just one trace (i.e., results of one well) but it is recommended to perform each dose level in triplicate.

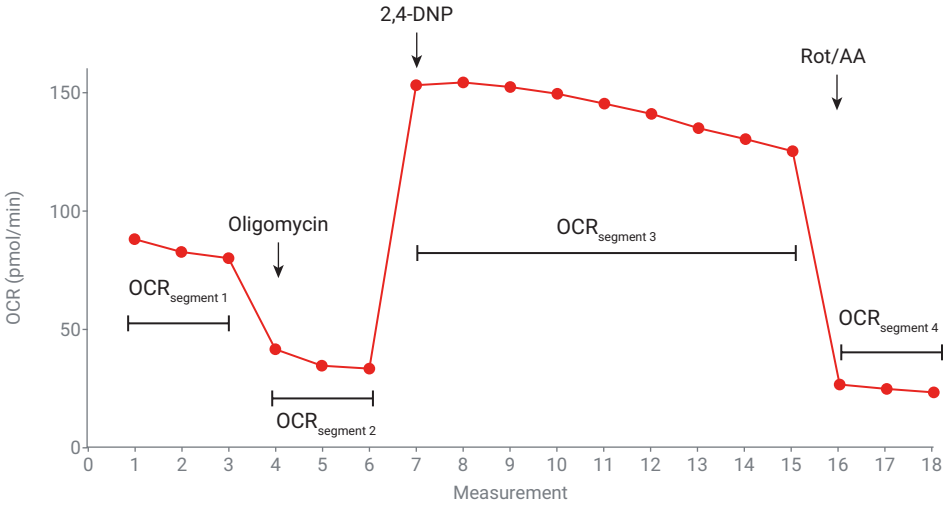

Figure 2. OCR time profiles in HepG2 cells (each measurement cycle included 3 minutes of measurement followed by 3 minutes of mixing). The cells were treated with oligomycin (after measurement 3), 2,4-DNP (after measurement 6), and Rot/AA (after measurement 15) and differed only in the treatment after measurement 6. Bold black brackets indicate the time segments 1, 2, 3, and 4 ($OCR_{segment\ 1}$, $OCR_{segment\ 2}$, $OCR_{segment\ 3}$, and $OCR_{segment\ 4}$).

The dynamic ranges of the controls were defined as the differences between the OCRs of two segments and were normalized to the basal respiration ($OCR_{segment1}$). For each MOA, the positive control $\Delta OCR_{positive\ control}$ was derived from the positive control and the negative control $\Delta OCR_{negative\ control}$ from unexposed cells in the same segment. The sample dynamic range was defined as the difference between the OCR of the negative control and the sample ΔOCR_{sample}.

OCR ratios for each MOA were then calculated using Equation 1 and the ΔOCR_i of the appropriate MOA. For more information on the OCR ratio calculations for each experimental design, see Müller, et al.[1]

Concentration-response curves were deduced to quantify the strength of the effect, expressed as EC_{50}, i.e., the concentration of chemical or sample that leads to 50% of the maximum effect of each MOA (Equation 2). If 50% of the effect was not reached, the concentration-response curves were evaluated with a linear model and EC_{10} derived.[4]

Equation 1.

$$OCR\ ratio_{MoA} = \frac{\Delta OCR_{sample} - \Delta OCR_{negative\ control}}{\Delta OCR_{positive\ control} - \Delta OCR_{negative\ control}}$$

Equation 2.

$$OCR\ ratio_{MoA} = \frac{100\%}{1 + 10^{slope(logEC_{50} - logconcentration)}}$$

Workflow 1: Inhibition of ATP synthase

As shown in Figure 3A, in each experiment, the basal respiration was measured for three cycles during Segment 1. Next, to assess ATP synthase inhibition, 2 µM tributyltin (TBT) was added to the cells in parallel with Agilent Seahorse XF assay medium (negative control, 0% inhibition) and oligomycin at 1 µM (positive control).

As shown in Figure 3A, the OCR dynamic range for ATP synthase inhibition is defined by 0% inhibition for cells that underwent addition of the Seahorse XF assay medium, and 100% inhibition for cells that underwent addition of 1 µM oligomycin. Because inhibition of the electron transport chain likewise decreases the OCR, the specificity of the inhibitory effect on ATP synthase was assessed using subsequent addition of 80 µM 2,4-DNP. If the cells exposed to the sample did not show an increase in OCR in segment 3 that was as high as the increase observed with the addition of 80 µM 2,4-DNP, it was concluded that the sample compound inhibits the electron transport chain, not ATP synthase. If the electron transport chain is disrupted, the electrochemical gradient cannot be built up and therefore cannot be uncoupled. As shown in Figure 3A, segment 2, it appears that TBT additionally inhibits the electron transport chain.

$\Delta OCR_{ATP\ synthesis\ inhibition}$ was calculated with Equation 3 and the ATP synthesis inhibition ratios were calculated using Equation 1.

Then, the concentration-response curve was derived by fitting $\Delta OCR_{ATP\ synthesis\ inhibition}$ as a function of the concentrations of the ATP synthase inhibitor with Equation 2 (Figure 3B).

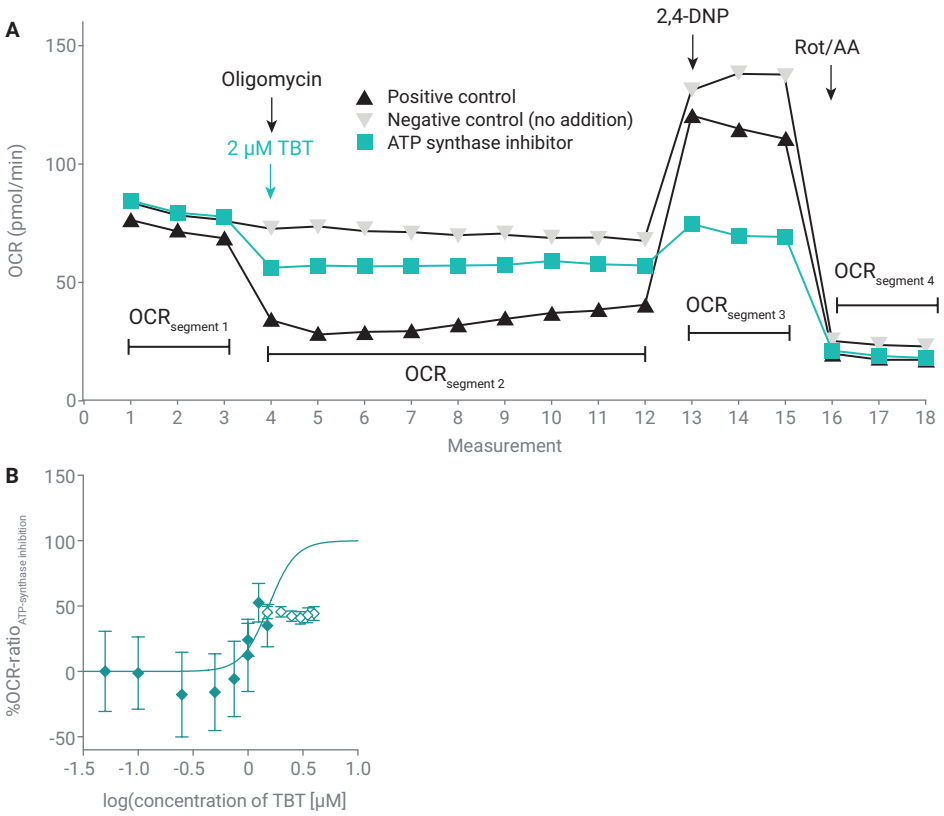

Figure 3. (A) OCR time profiles in HepG2 cells for ATP synthase inhibition. The OCR profile of the positive control (injection of oligomycin, followed by 2,4-DNP, then Rot/AA) is illustrated as black upward-pointing triangles. The OCR profile of the negative control is displayed as empty downward-pointing triangles. The sample that acts as an inhibitor of ATP synthase (2 µM tributyltin, TBT) is shown as green squares. (B) Concentration-response curve of TBT, an inhibitor of ATP synthase. The three higher concentrations were overdosed and excluded from the concentration-response curve (Equation 2).

Equation 3.

$$\Delta OCR_{ATP\ synthesis\ inhibition} = \frac{OCR_{segment\ 1} - OCR_{segment\ 2}}{OCR_{segment\ 1}}$$

Workflow 2: Uncoupling of oxidative phosphorylation

To measure mitochondrial proton gradient uncoupling, the OCR was reduced by the addition of 1 µM oligomycin (100% inhibition) after measurement of the basal respiration rate for three cycles in Segment 1. This Segment 2 step increased the dynamic range of OCR inhibition (Figure 4A). At Segment 3, 80 µM 2,4-DNP was added to the positive controls, Seahorse XF assay medium was added to the negative controls, the sample was added, and the OCR was measured for nine cycles over 54 minutes. Parallel addition of 80 µM 2,4-DNP established the upper effect level (positive control, black line) and media established the no-effect level (negative control, green line) as shown in Figure 4A. At Segment 4, 0.5 µM Rot/AA was added and the OCR was measured for three cycles.

Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) or 2,4-DNP uncouple the proton gradient by allowing protons to pass the membrane barrier without ATP production. Although 2,4-DNP was used as the reference uncoupler in this experiment, FCCP, available as part of the Agilent Seahorse XF Cell Mito Stress Test Kit, is a widely recognized alternative that can be used for this workflow.

Equation 4.

$$\Delta OCR_{uncoupling} = \frac{OCR_{segment\ 3} - OCR_{segment\ 2}}{OCR_{segment\ 1}}$$

Quantification of uncoupling used the same type of experiment as described earlier but as shown in Figure 4A, the positive control for uncoupling 2,4-DNP was replaced by the sample. $\Delta OCR_{uncoupling}$ was calculated with Equation 4 and the mitochondrial membrane uncoupling ratios were calculated using the common approach. Figure 4B shows the concentration-response curve of a typical uncoupler and the derived EC_{50}. As the highest concentration led to quenching of the signal, the evaluation of the low-effect level, linear concentration-response curve is an alternative as described in more detail in Müller, *et al.*[1]

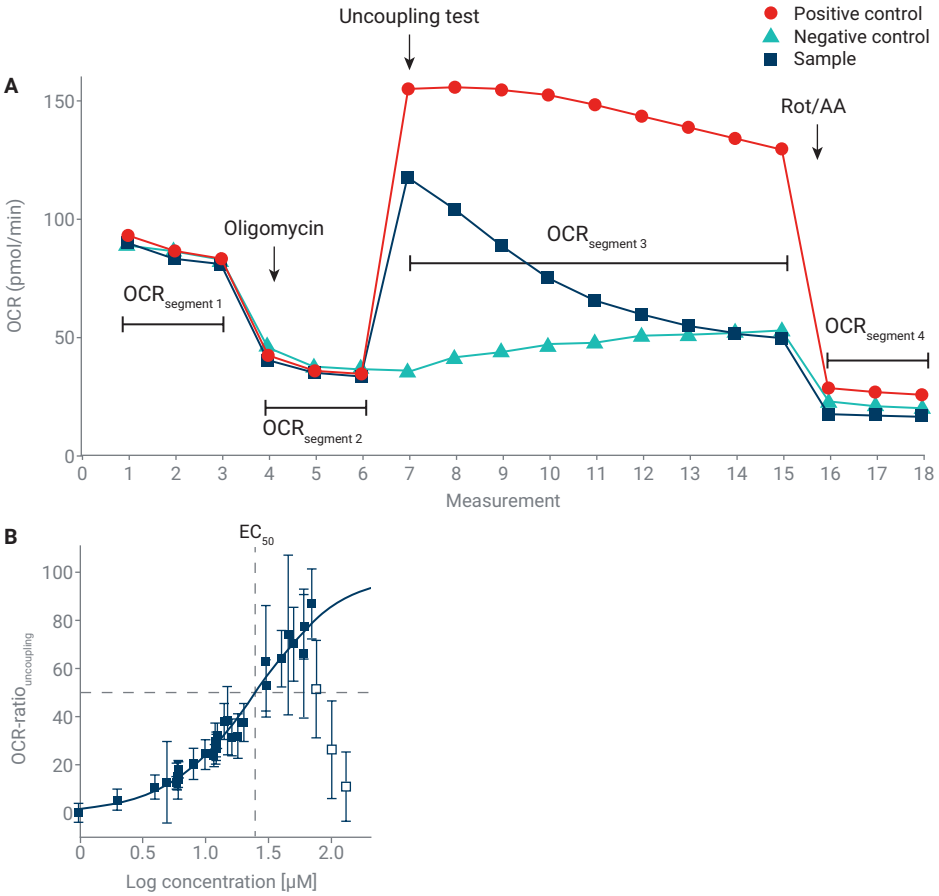

Figure 4. (A) OCR time profiles in HepG2 cells for uncoupling. The OCR profile of the positive control (injection of oligomycin, followed by 2,4-DNP, and then Rot/AA) is illustrated as red dots. The OCR profile of the negative control is displayed as green triangles. The sample that acts as an uncoupler (example bromoxynil) is shown as blue squares. (B) Concentration-response curve of an uncoupler (bromoxynil). The three higher concentrations were overdosed and excluded from the concentration-response curve (Equation 2).

Workflow 3: Inhibition of the electron transport chain

A mix of rotenone and antimycin A (Rot/AA) was used as reference compounds to disrupt the electron transport chain. To measure electron transport chain inhibition, 1 µM oligomycin was added to the cells after basal respiration rate measurement to ensure that any OCR decrease after sample addition was caused by ATP synthase inhibition (Segment 2). After three measurement cycles, 80 µM 2,4-DNP was added to bring up the OCR and thus increase the dynamic range. The OCR was then measured for an extra three cycles (Segment 3). In Segment 4, the cells were exposed either to the sample, to 0.5 µM Rot/AA, or Seahorse XF assay medium (Figure 5A). The decreased OCR caused by adding Rot/AA represents 100% electron transport chain inhibition (positive control, black line in Figure 5A), and the OCR after media addition marks the no-effect level (negative control, empty downwards pointing triangles in Figure 5A).

$\Delta OCR_{e^-\text{-transport inhibition}}$ was calculated with Equation 5 and the electron transport chain inhibition ratios were calculated, yielding the concentration-effect curve shown in Figure 5B for the example of azoxystrobin.

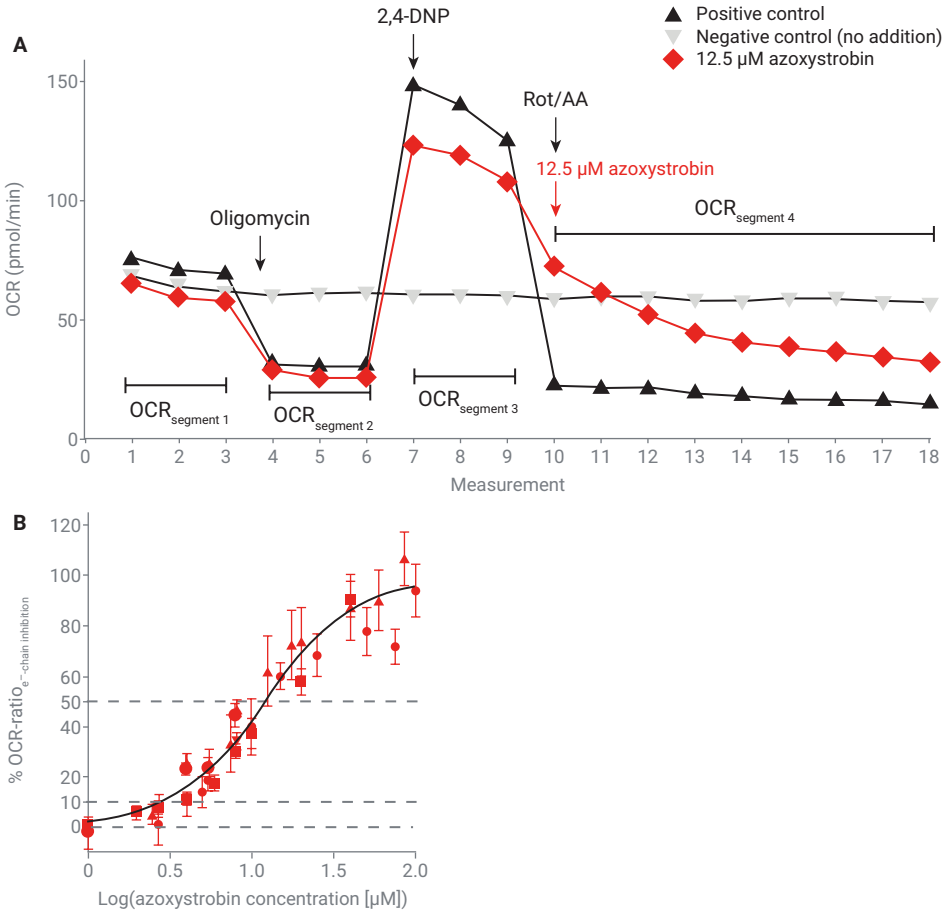

Figure 5. (A) OCR time profiles in HepG2 cells for electron transport chain inhibition. The OCR profile of the positive control (injection of oligomycin, followed by 2,4-DNP, and then Rot/AA) is illustrated as black upward-pointing triangles. The OCR profile of the negative control is displayed as empty downward-pointing triangles. (B) Concentration-response curve of an inhibitor of the electron transport chain (red symbols, different symbols refer to independent experiments). The black line is the concentration-response curve (Equation 2).

Equation 5.

$$\Delta OCR_{e^-\text{-transport inhibition}} = \frac{OCR_{segment\ 3} - OCR_{segment\ 4}}{OCR_{segment\ 1}}$$

Application of the method to chemical assessments

The sample compounds evaluated were known and regulated environmental pollutants often found in surface waters, namely the herbicide bromoxynil and the fungicides azoxystrobin and pyraclostrobin as detailed in Müller, *et al.*[1]

A summary of the effect of the various concentrations of the chemicals tested on OCRs is provided in Table 1. The EC_{10} values determined from the linear portion of the CRC were within the 95% confidence interval of the EC_{10} derived from the sigmoidal logarithmic CRC. The excellent reproducibility of five independent repetitions is indicated by the low standard deviation, confirming that the linear CRC evaluation was the appropriate approach. Results obtained for bromoxynil, 2,4-DNP, azoxystrobin, and pyraclostrobin showed high precision and reproducibility, confirming the experiments using the Seahorse XFe96 Analyzer were applicable to the assessment of individual chemicals.

Organotin compounds such as TBT were widely used as biocides, especially as antifouling paintings on ships. They interfere with the oxidative phosphorylation in mitochondria in several ways and inhibit electron transport, uncouple the mitochondrial membrane, and inhibit ATP-synthase.

TBT was tested using workflow 1 for ATP synthase inhibition relative to 1 µM oligomycin (Figure 6A, left Y-axis in red). At concentrations higher than 2 µM a maximum effect level of around 50% appears to be reached, and not exceeded when concentrations were further increased. If TBT was tested in workflow 3, a distinct inhibition of the electron transport chain was also observed (Figure 6A, right Y-axis in blue). If the concentration-response curves for inhibition of the ATP synthase and the electron transport chain were directly compared (Figure 6A), they had the same shape with a sigmoidal curve at a lower effect level and a plateau above 2 µM TBT.

TBT was also tested for uncoupling activity using workflow 2. The OCR $ratio_{uncoupling}$ decreased in a concentration-dependent manner (right, Y-axis, teal data points in Figure 6B). Even at TBT concentrations that already reached a plateau of OCR $ratio_{ATP\text{-}synthase\ inhibition}$ at 50%, the OCR $ratio_{uncoupling}$ continued to decrease with increasing TBT concentrations.

Table 1. Summary of the EC_{10} values for single chemicals calculated from the sigmoidal-log CRC and linear portion of the CRC.

Sample	Active endpoint	EC_{10} [µM] (Sigmoidal-log CRC)	EC_{10} [µM] (Linear CRC)
2,4-Dinitrophenol	%OCR $ratio_{uncoupling}$	9.93 (95% CI: 7.7 – 12.4)	6.46 ±0.83
Bromoxynil	%OCR $ratio_{uncoupling}$	4.53 (95% CI: 3.9 – 5.2)	4.06 ±0.09
Azoxystrobin	%OCR $ratio_{e^-\text{-}chain\ inhibition}$	2.79 (95% CI: 2.2 – 3.4)	2.61 ±0.13
Pyraclostrobin	%OCR $ratio_{e^-\text{-}chain\ inhibition}$	1.73 (95% CI: 1.6 – 1.8)	1.18 ±0.11
Tributyltin	%OCR $ratio_{e^-\text{-}chain\ inhibition}$	0.50 (95% CI: 0.1 – 1.1)	1.38 ±0.26
Tributyltin	%OCR $ratio_{ATP\text{-}synthase\ inhibition}$	0.84 (95% CI: 0.5 – 1.2)	0.71 ±0.33

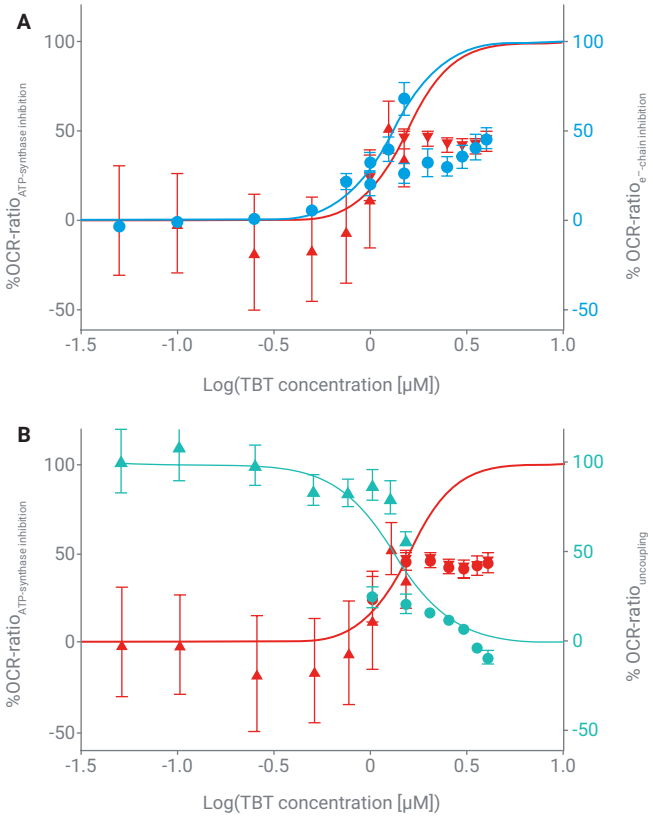

Figure 6. (A) Concentration-response curves of TBT for ATP synthase inhibition (left axis, red triangles) and inhibition of the electron transport chain (right Y-axis, blue circles). (B) Concentration-response curves of TBT for ATP synthase inhibition (left axis, red triangles and circles, different symbols refer to independent experiments) in comparison to uncoupling (right Y-axis, teal triangles and circles, different symbols refer to independent experiments).

The example of TBT illustrates that it is possible to differentiate between uncoupling and inhibition of the electron transport chain using the different workflows of OCR measurements but it is not possible to clearly say if the ATP synthase or the electron transport chain were inhibited.

Application to environmental samples

Water samples from the Ammer River in Germany that were extracted using solid-phase extraction (SPE) were also tested.

The relative enrichment factor (REF) of the sample was calculated from Equation 6 as the ratio of the enrichment factor (EF) from the SPE extraction and concentration step and the dosing factor (DF) of the sample in the bioassay. The enrichment factor can be calculated using Equation 7 and the dosing factor with Equation 8.

Most extracts showed no activity, but one extract (water extract A, Figure 7A) showed low activity for uncoupling but no other effects. As the effect did not exceed 30% of the concentration, the response curve was linear and EC_{10} was derived. The EC_{10} for uncoupling was at a REF of 97.9 ±12.4 $L_{water}/L_{bioassay}$, which means that the water sample had to be enriched almost 100 fold to trigger a 10% effect.

Another water extract was not active in OCR $ratio_{uncoupling}$ but showed a distinct linear concentration-response curve for OCR $ratio_{e\text{-chain inhibition}}$ (water extract B, Figure 7B). Again, the samples had to be enriched to show any effect. The EC_{10} for inhibition of the electron transport chain derived from the linear portion of the concentration-response curve was REF 9.3 ±1.8, that is the sample had to be enriched 9.3 times to reach 10% effect. This is a low but not negligible effect.

Equation 6.

$$REF = \frac{\text{mass or volume extracted}}{\text{final volume in bioassay}} = EF \cdot DF\left(\frac{kg_{matrix} \text{ or } L_{water}}{L_{bioassay}}\right)$$

Equation 7.

$$EF = \frac{\text{mass or volume extracted}}{\text{final volume of extract}} \left(\frac{kg_{matrix} \text{ or } L_{water}}{L_{extract}}\right)$$

Equation 8.

$$DF = \frac{\text{volume of extract dosed}}{\text{final volume in bioassay}} \left(\frac{L_{extract}}{L_{bioassay}}\right)$$

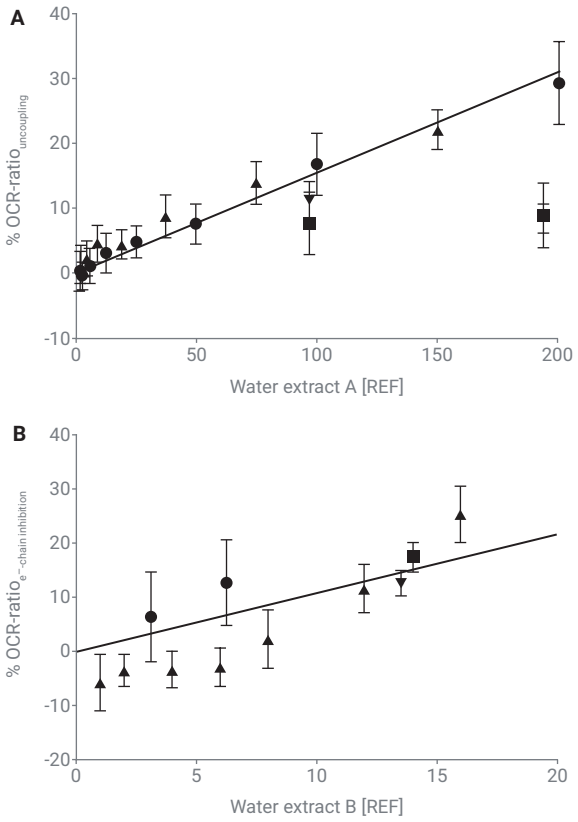

Figure 7. (A) The concentration-response curve for OCR ratiouncoupling of water extract A. (B) The concentration-response curve for OCR $ratio_{e^-\text{-chain inhibition}}$ of water extract B. REF = relative enrichment factor in units of $L_{water}/L_{bioassay}$. Different symbols refer to independent experiments.

Experimental details of Seahorse XFe96 Analyzer method

The Seahorse XFe96 sensor cartridges were incubated in sterile water overnight at 37 °C. At least 1 hour before the experiments, the sterile water was exchanged with XF calibrant solution, and the sensor cartridge was incubated under the same conditions until the measurement. The medium was exchanged with the Seahorse XF assay medium before the measurement. The culture medium was aspirated, leaving 20 µL in each well. Each well was refilled to a final volume of 180 µL with Seahorse XF assay medium supplemented with 10 mM glucose, 1 mM pyruvate, 2 mM glutamine, and 5 mM HEPES and adjusted to pH 7.4 (see Table 2 for recommended media). This step was repeated three times. The microplate was then incubated at 37 °C for 1 to 2 hours.

In every experiment, a reference (positive control) profile was obtained by injecting the reference chemicals oligomycin (10 µM), 2,4-DNP (0.8 mM), and Rot/AA (5 µM) as follows: port A→port B→port C. The positive controls were prepared and injected at 10x concentrations to cause complete ATP synthase inhibition, 95% uncoupling, and complete electron transport chain inhibition, serving as the reference to which all samples were compared.

FCCP, available as part of the Agilent Seahorse XF Cell Mito Stress Test Kit, is a widely recognized alternative to the reference uncoupler 2,4-DNP that was used in this work. Details about this kit and the Agilent recommended materials for this assay can be found in the Recommended Materials section of this application note.

Samples and controls were added at the beginning of each time segment (Figure 2), depending on the experiment performed. OCR traces were generated by lowering the sensor probes and measuring the OCR for 3 minutes, followed by aeration for 3 minutes and mixing following the addition of the reference compounds. Traces were then compared in parallel measurements, where one of the reference compounds was replaced by the sample or by a negative control.

To characterize the potency of a chemical to inhibit mitochondrial ATP synthase, the chemical or sample of interest was loaded into port A at the desired concentration instead of oligomycin. To evaluate potency to uncouple the mitochondrial membrane, the sample was loaded into port B at the desired concentration instead of 2,4-DNP. To assess the potency to inhibit the mitochondrial electron transport chain, the sample was loaded into port C instead of Rot/AA. Because the OCR reverted to basal respiration over time, a negative control was added on the same plate. For the negative control, the Seahorse XF Assay Medium was loaded into port A, port B, or port C instead of oligomycin, 2,4-DNP, or Rot/AA, respectively. The plate included the treatments positive control (port A, oligomycin; port B, 2, 4-DNP; port C, Rot/AA), negative control (medium filled into port A, B or C instead of oligomycin, 2,4-DNP, or Rot/AA, respectively), sample (loaded into either port A, B, or C instead of oligomycin, 2,4-DNP, or Rot/AA, respectively), background (wells containing no cells but treated the same as the positive control), and medium background (medium loaded into ports A, B, and C). After injection of the sample and the positive and negative controls in parallel, the OCR was measured in nine measurement cycles over 54 minutes to provide enough time for the sample to enter the cells and affect mitochondrial function.

Conclusion

The Seahorse XFe96 Analyzer OCR workflow was determined to be applicable to the identification of mitochondrial toxicity in environmental water matrices. The results from analysis of single chemicals with known mechanisms of mitochondrial toxicity validated the workflow. Results from analysis of Ammer River samples showed that the workflow was able to identify uncoupling effects at sampling site 1 and electron transport inhibition at sample site 2. One interpretation of the results is that a tributary between the two sites introduced a fungicide acting as electron transport inhibitor. This conclusion would have to be confirmed using chemical analyses. Overall, high throughput EDA using the Seahorse XFe96 Analyzer offers a rapid and cost-effective tool to enhance the understanding of the occurrence and biological activity of chemicals in environmental samples.

Recommended materials

Table 2. Recommended materials.

Part number	Description
	Agilent Seahorse XFe96 Analyzer
103729-100	Agilent Seahorse XFe96 FluxPak Mini (PDL microplates) Includes six XFe96 sensor cartridges, six XFe96 precoated poly-D-lysine (PDL) cell culture microplates, and one bottle of XF calibrant solution 500 mL
103015-100	Agilent Seahorse XF Cell Mito Stress kit Includes six single-use pouches each contains one tube of oligomycin, FCCP, and rotenone/antimycin A
103575-100	Agilent Seahorse XF DMEM medium, pH 7.4, 500 mL
103577-100	Agilent Seahorse XF 1.0 M glucose solution, 50 mL
103578-100	Agilent Seahorse XF 100 mM pyruvate solution, 50 mL
103579-100	Agilent Seahorse XF 200 mM glutamine solution, 50 mL

Abbreviations

2,4-DNP: 2,4-dinitrophenol

ATP: Adenosine triphosphate

DF: Dosing factor

EC_{10}: Effective concentration at 10% inhibition

EC_{50}: Half maximal effective concentration

EF: Enrichment factor

ETC: Electron transport chain

FCCP: Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone

LC/Q-TOF: Liquid chromatography/quadrupole TOF

MOA: Mode of action

OCR: Oxygen consumption rate

REF: Relative enrichment factor

Rot/AA: Rotenone and antimycin A

SPE: Solid-phase extraction

TBT: Tributyltin

References

1. Müller, M. *et al.* Mitochondrial Toxicity of Selected Micropollutants, Their Mixtures, and Surface Water Samples Measured by the Oxygen Consumption Rate in Cells. *Environ. Toxicol. Chem*. **2019**, *38*, 1000–1011.
2. Papkovsky *et al.* Development of a Novel Environmental Monitoring System based on Optical Oxygen Sensing and Respirometry, (AT-04-01-01) EPA IE, **2009**.
3. Sobus *et al.* Integrating Tools for Non-Targeted Analysis Research and Chemical Safety Evaluations at the US EPA. *Journal of Exposure Science & Environmental Epidemiology* **2018**, 28, 411–426.
4. Escher, B. *et al.* The Advantages of Linear Concentration-Response Curves for In Vitro Bioassays with Environmental Samples. *Environmental Toxicology and Chemistry* **2018**, *37(9)*, 2273–2280.

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