# ADDITIONAL MATERIALS AVAILABLE ON THE HEI WEBSITE

## Research Report 197

# Cellular and Acellular Assays for Measuring Oxidative Stress Induced by Ambient and Laboratory-Generated Aerosols

**Ng et al.**

## Additional Materials 2
## Appendix B: Cellular Assay Optimization

Note: Additional Materials may appear in a different order than in the original Investigators' Report, and some remnants of their original names may be apparent. HEI has not changed the content of these documents, only their numeric identifiers. Additional Materials 2 was originally Appendix B.

These Additional Materials were not formatted or edited by HEI. This document was part of the HEI Review Committee's review process.

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## Appendix B: Cellular assay optimization

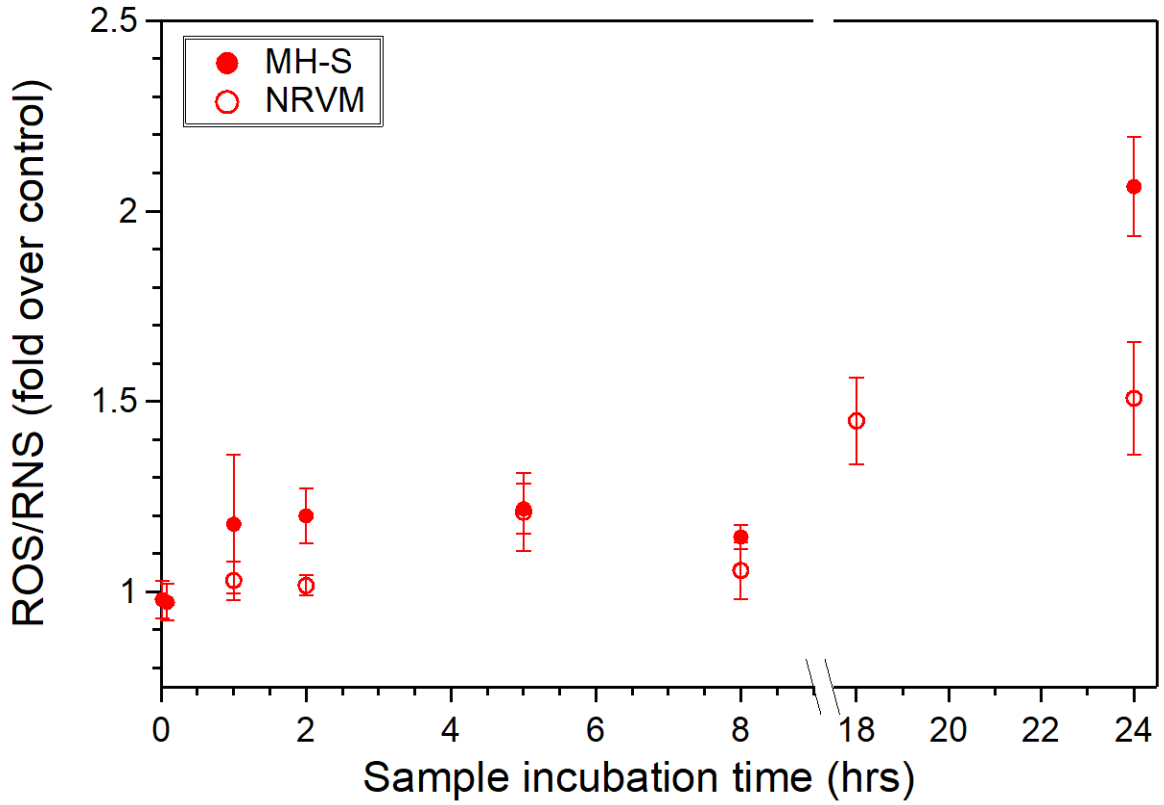



**Figure B1.** ROS/RNS produced as a result of exposure to bacterial cell wall component, lipopolysaccharide (LPS), for various incubation times. Two cell types, macrophages (MH-S, closed markers) and cardiomyocytes (NRVM, opened markers), were investigated. ROS/RNS production is expressed as a fold increase over probe-treated cells exposed to stimulant-free media (negative control). At 24 hrs the normalized positive control response is the highest for both cell types.

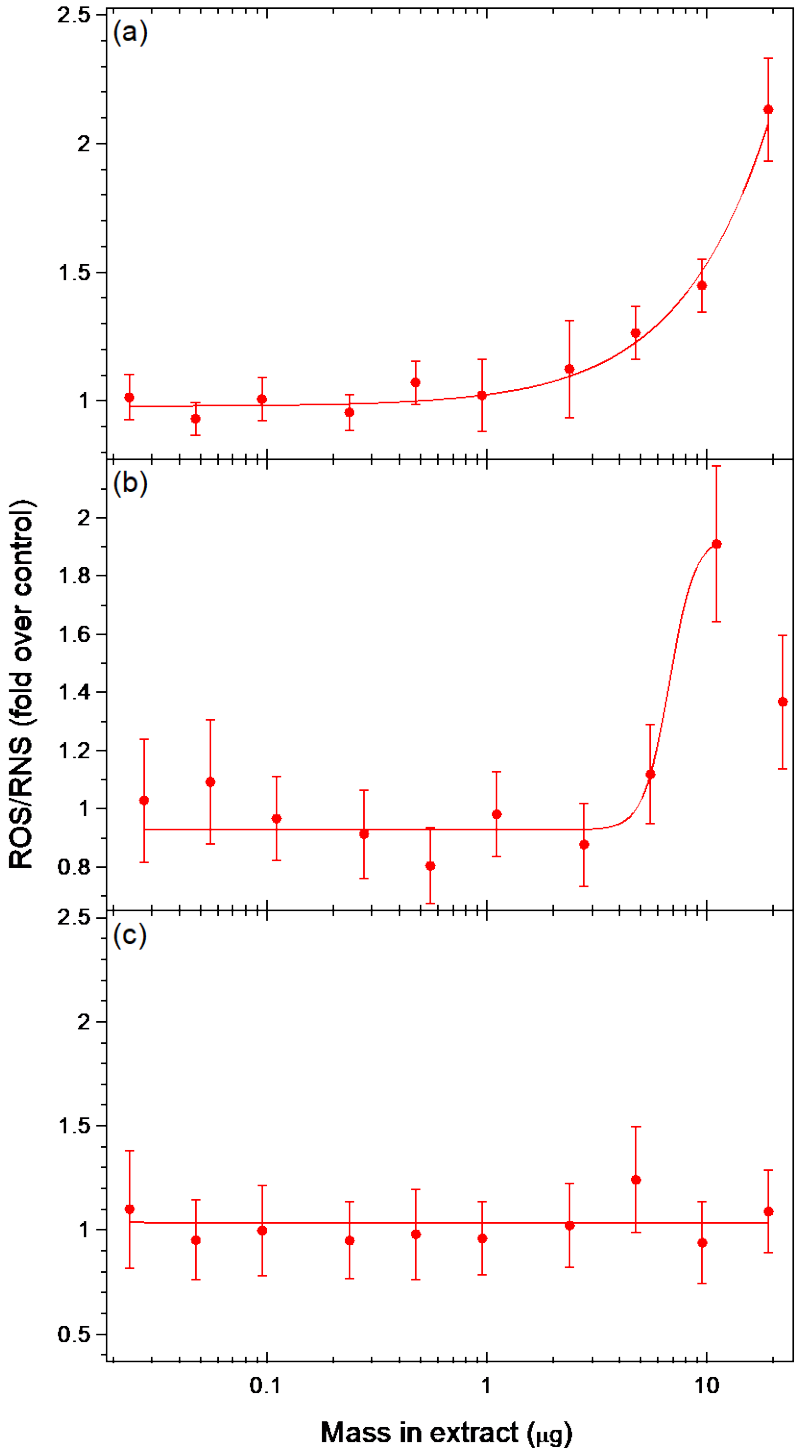



**Figure B2.** Non-classical dose-response behaviors for ROS/RNS produced as a result of PM exposure observed in this study. Briefly, these behaviors include: a) samples where the maximum response was not attained, (b) samples where a decreased response was observed at higher doses, and (c) samples where no response above the baseline was observed over the dose range investigated.

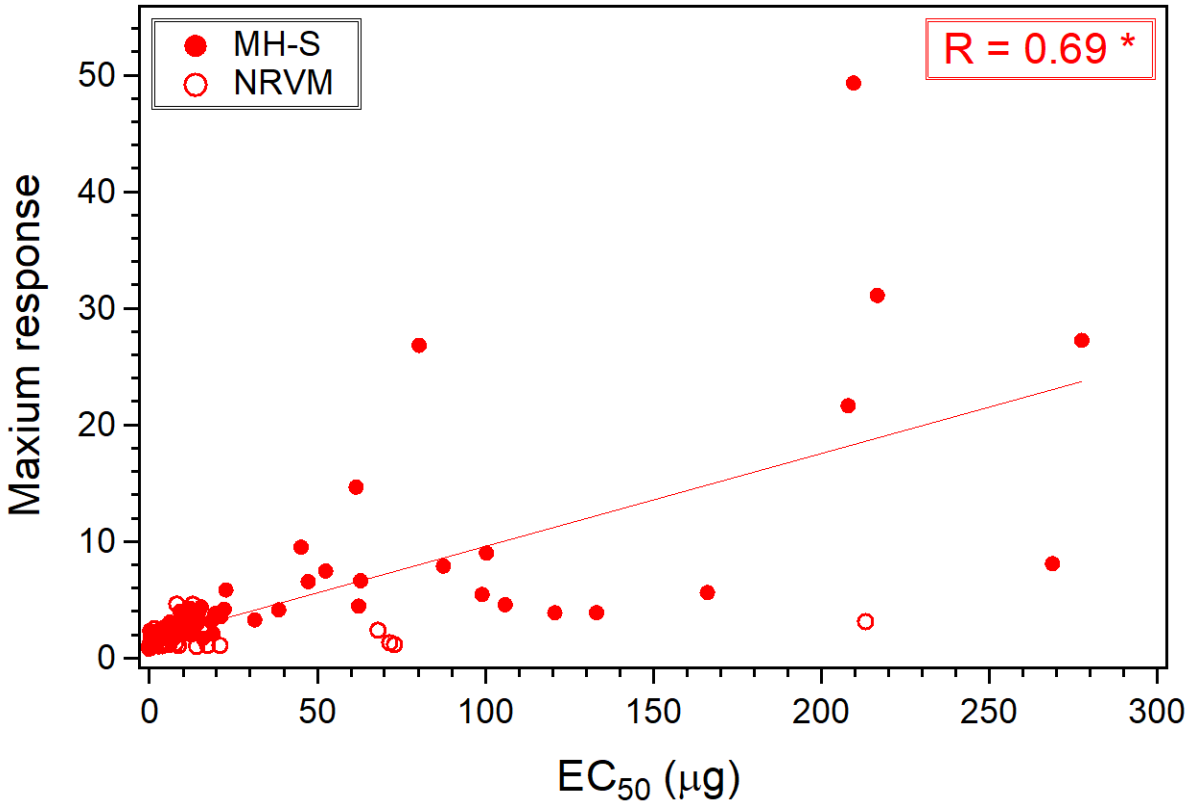



**Figure B3.** Correlation between maximum ROS/RNS production and $EC_{50}$ for ambient filter samples analyzed using both macrophages (MH-S, closed markers) and cardiomyocytes (NRVM, opened markers). Data points represent individual filter samples. A simple linear regression and the corresponding Pearson's coefficient are shown. * indicates significance, $p < 0.05$.

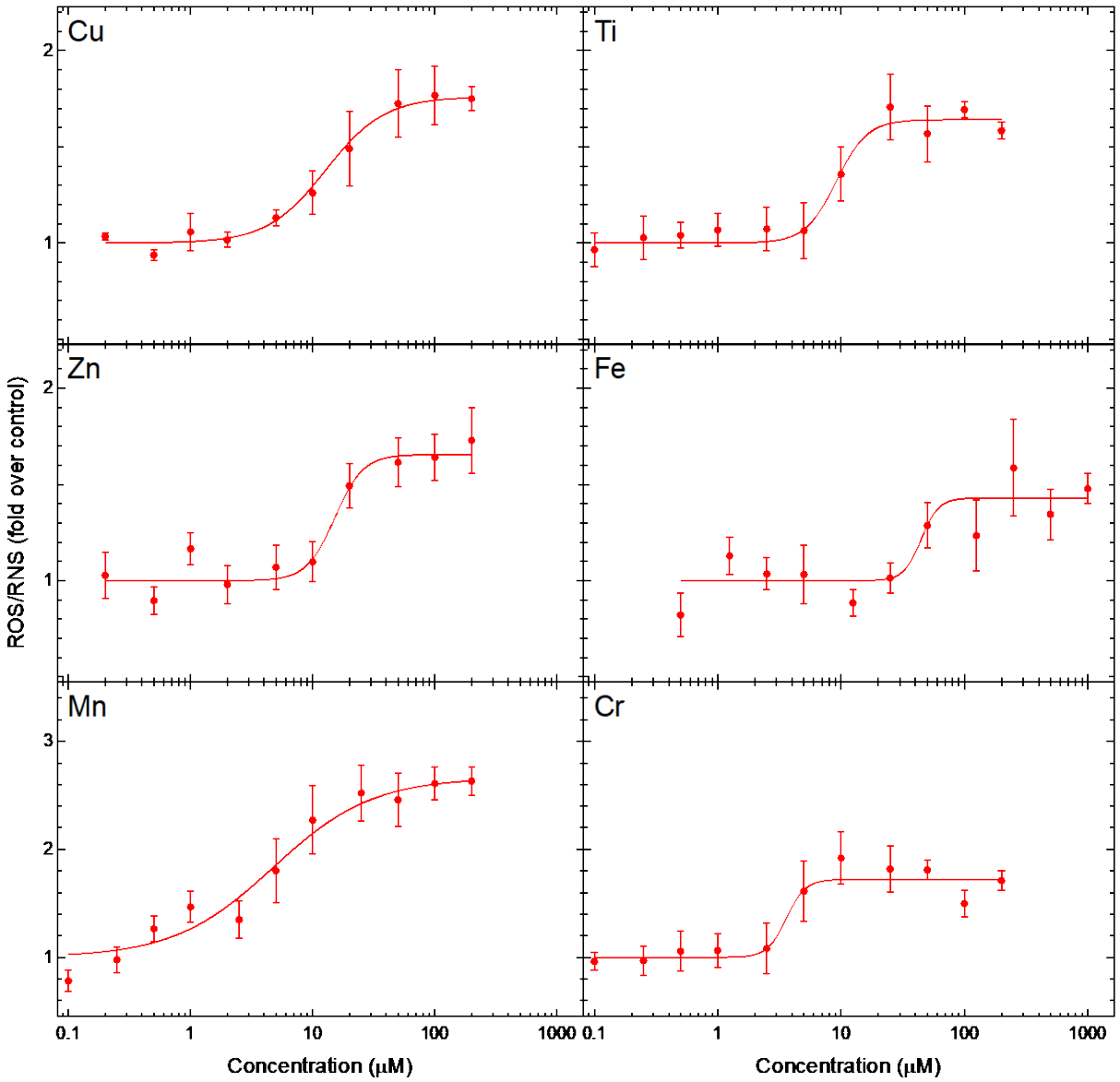



**Figure B4.** ROS/RNS produced as a result of exposure to pure metal salt solutions: copper ($Cu(II)SO_4$), titanium ($C_6K_2O_{12}Ti$), zinc ($ZnCl_2$), iron ($Fe(II)SO_4$), manganese ($Mn(II)SO_4$), and chromium ($Cr(III)Cl_3$). Dose ranges span the water-soluble metal concentrations observed in ambient samples collected as part of the Southeastern Center for Air Pollution and Epidemiology (SCAPE) study.

**Table B1.** Within plate and overall standard errors for positive and negative controls assessed in the cellular ROS/RNS assay.

| Control | Standard error (%) | |
|---|---|---|
| | Plate | Overall |
| Probe-treated control | 9.6 | 4.0 |
| Blank filter extract | 11 | 4.6 |
| LPS | 11 | 4.0 |
| $H_2O_2$ | 14 | 6.7 |
| Reference filter extract | 11 | 4.0 |

**Table B2.** Pearson's correlation coefficients for linear regressions between extrinsic and intrinsic oxidative potentials as measured by dithiothreitol (DTT activity) and dose-response metrics, including maximum response, $EC_{50}$, Hill slope, and threshold.

| | Maximum response | $EC_{50}$ | Hill slope | Threshold |
|---|---|---|---|---|
| **Extrinsic DTT activity** | 0.16 | 0.19 | -0.036 | 0.14 |
| **Intrinsic DTT activity** | -0.075 | -0.069 | 0.059 | 0.076 |

**Table B3.** Pearson's correlation coefficients for linear regressions between absolute concentrations of various PM constituents and dose-response metrics, including maximum response, $EC_{50}$, Hill slope, threshold, and extrinsic AUC. Water-soluble PM constituents, $PM_{2.5}$ concentrations, and metals grouped by source apportionment results (brake/tire wear: Ti, Cu, Zn, Ba; biomass burning: K, As, Br, Pb; secondary formation: S, Fe, Se; and mineral dust: Ca, Mn, Sr) are included.

| | Maximum response | $EC_{50}$ | Hill slope | Threshold | AUC |
|---|---|---|---|---|---|
| **Water-soluble organic carbon** | 0.18 | 0.087 | -0.11 | -0.054 | 0.39 |
| **Brown carbon** | 0.090 | 0.26 | -0.048 | 0.12 | -0.027 |
| **Ti** | -0.024 | -0.12 | 0.048 | -0.11 | 0.38 |
| **Cu** | -0.037 | -0.11 | 0.19 | -0.096 | 0.21 |
| **Zn** | 0.051 | 0.020 | 0.025 | -0.0088 | 0.23 |
| **Ba** | -0.072 | -0.092 | 0.14 | -0.13 | 0.17 |
| **Brake/tire wear** | 0.013 | -0.050 | 0.14 | -0.060 | 0.26 |
| **K** | 0.13 | 0.20 | -0.049 | 0.12 | 0.15 |
| **As** | 0.021 | 0.12 | 0.11 | 0.12 | -0.022 |
| **Br** | 0.091 | 0.12 | -0.094 | 0.038 | 0.25 |
| **Pb** | 0.090 | 0.13 | -0.075 | 0.13 | 0.15 |
| **Biomass burning** | 0.14 | 0.22 | -0.028 | 0.13 | 0.13 |
| **S** | 0.24 | 0.12 | -0.012 | -0.015 | 0.28 |
| **Fe** | 0.11 | -0.022 | 0.036 | -0.13 | 0.43 |
| **Se** | 0.20 | 0.20 | 0.020 | 0.14 | 0.22 |
| **Secondary formation** | 0.23 | 0.10 | -0.029 | -0.038 | 0.35 |
| **Ca** | 0.20 | -0.013 | -0.10 | -0.15 | 0.15 |
| **Mn** | 0.23 | 0.091 | -0.12 | -0.16 | 0.38 |
| **Sr** | 0.096 | -0.026 | 0.28 | -0.11 | 0.16 |
| **Mineral dust** | 0.20 | -0.020 | -0.10 | -0.13 | 0.22 |
| **Cr** | -0.040 | -0.049 | -0.11 | -0.11 | -0.094 |
| **V** | 0.12 | 0.061 | -0.23 | -0.087 | 0.18 |
| **Total metals** | 0.24 | 0.090 | -0.044 | -0.053 | 0.36 |
| **$PM_{2.5}$** | 0.29 | 0.20 | -0.052 | 0.014 | 0.30 |

**Table B4.** Pearson's correlation coefficients for linear regressions between mass fractions of various PM constituents and dose-response metrics, including maximum response, $EC_{50}$, Hill slope, threshold, and intrinsic AUC. Water-soluble PM constituents and metals grouped by source apportionment results (brake/tire wear: Ti, Cu, Zn, Ba; biomass burning: K, As, Br, Pb; secondary formation: S, Fe, Se; and mineral dust: Ca, Mn, Sr) are included.

| | Maximum response | $EC_{50}$ | Hill slope | Threshold | AUC |
|---|---|---|---|---|---|
| **Water-soluble organic carbon** | -0.013 | -0.052 | -0.071 | -0.072 | 0.14 |
| **Ti** | -0.13 | -0.21 | 0.17 | -0.12 | 0.42 |
| **Cu** | -0.079 | -0.17 | 0.29 | -0.095 | 0.14 |
| **Zn** | -0.10 | -0.17 | 0.074 | -0.047 | 0.38 |
| **Ba** | -0.13 | -0.17 | 0.20 | -0.16 | 0.32 |
| **Brake/tire wear** | -0.014 | -0.043 | 0.19 | -0.0037 | 0.020 |
| **K** | -0.052 | 0.044 | 0.064 | 0.12 | 0.18 |
| **As** | -0.056 | 0.011 | 0.19 | 0.087 | 0.050 |
| **Br** | -0.10 | -0.068 | -0.083 | 0.021 | 0.11 |
| **Pb** | -0.082 | -0.026 | -0.049 | 0.15 | -0.032 |
| **Biomass burning** | 0.030 | 0.18 | 0.11 | 0.20 | -0.20 |
| **S** | -0.029 | -0.12 | -0.030 | -0.099 | 0.16 |
| **Fe** | -0.025 | -0.15 | 0.076 | -0.18 | 0.19 |
| **Se** | 0.028 | 0.084 | 0.084 | 0.25 | -0.32 |
| **Secondary formation** | -0.036 | 0.041 | -0.034 | 0.033 | -0.18 |
| **Ca** | -0.045 | -0.19 | -0.082 | -0.15 | 0.46 |
| **Mn** | -0.018 | -0.054 | -0.10 | -0.20 | 0.54 |
| **Sr** | -0.035 | -0.097 | 0.35 | -0.11 | 0.17 |
| **Mineral dust** | 0.029 | -0.14 | -0.056 | -0.14 | 0.30 |
| **Cr** | -0.12 | -0.12 | -0.14 | -0.11 | -0.086 |
| **V** | -0.083 | -0.067 | -0.17 | -0.11 | 0.29 |

**Table B5.** Rough dose estimation for dose delivered to alveolar macrophages assuming 100% deposition and one day exposure.

| | |
|---|---|
| **Alveolar ELF volume** | 9 mL |
| **Average inhalation rate (Cross et al. 1998)** | 18 $m^3$/day |
| **Average PM concentration (Atlanta)** | 10 µg/$m^3$ |
| **Total PM inhaled per day (Pope et al. 2011)** | 180 µg/day |
| **PM concentration in alveolar ELF** | 0.02 µg/µL |
| **PM mass exposed to 20,000 cells** | 2.4 µg |

**Intrinsic and extrinsic conversion calculations:**

| Case | PM concentration | Collection flow rate | Collection time | PM mass collected |
|---|---|---|---|---|
| | µg $m^{-3}$ | $m^3$ $hr^{-1}$ | hr | µg |
| 1 | 10 | 1 | 1 | 10 |
| 2 | 20 | 1 | 1 | 20 |

Sample calculation for PM mass collected:

Case 1:

$$PM\ concentration = 10\ \frac{\mu g}{m^3}$$

$$PM\ mass\ collected = 10\ \frac{\mu g}{m^3}\ x\ 1\frac{m^3}{hr} x1\ hr = 10\ \mu g$$

Case 2:

$$PM\ concentration = 20\ \frac{\mu g}{m^3}$$

$$PM\ mass\ collected = 20\ \frac{\mu g}{m^3}\ x\ 1\frac{m^3}{hr} x1\ hr = 20\ \mu g$$

| Case | PM mass collected | Extraction volume | 1*x* exposure volume | 1*x* concentration |
|---|---|---|---|---|
| | µg | mL | µL | µg |
| 1 | 10 | 1 | 100 | 1 |
| 2 | 20 | 2 | 100 | 1 |

Sample calculation for 1*x* concentration:

Case 1:

$$1x\ concentration = \frac{10\ \mu g}{1\ mL} x\ \frac{1\ mL}{1000\ \mu L} x\ 100\ \mu L = 1\ \mu g$$

Case 2:

$$1x\ concentration = \frac{20\ \mu g}{2\ mL} x\ \frac{1\ mL}{1000\ \mu L} x\ 100\ \mu L = 1\ \mu g$$

| Case | $1x$ concentration<br>µg | PM concentration<br>µg $m^{-3}$ | AUC<br>fold | $AUC_{intrinsic}$<br>$µg^{-1}$ | $AUC_{extrinsic}$<br>$m^{-3}$ |
|---|---|---|---|---|---|
| 1 | 1 | 10 | 3 | 3 | 30 |
| 2 | 1 | 20 | 3 | 3 | 60 |

Sample calculation for intrinsic and extrinsic AUC:

Case 1:

$$AUC_{intrinsic} = \frac{3}{1\ \mu g} = \frac{3}{\mu g}$$

$$AUC_{extrinsic} = AUC_{intrinsic}\ x\ PM\ concentration$$

$$AUC_{extrinsic} = \frac{3}{\mu g}\ x\ 10\ \frac{\mu g}{m^3} = \frac{30}{m^3}$$

Case 2:

$$AUC_{intrinsic} = \frac{3}{1\ \mu g} = \frac{3}{\mu g}$$

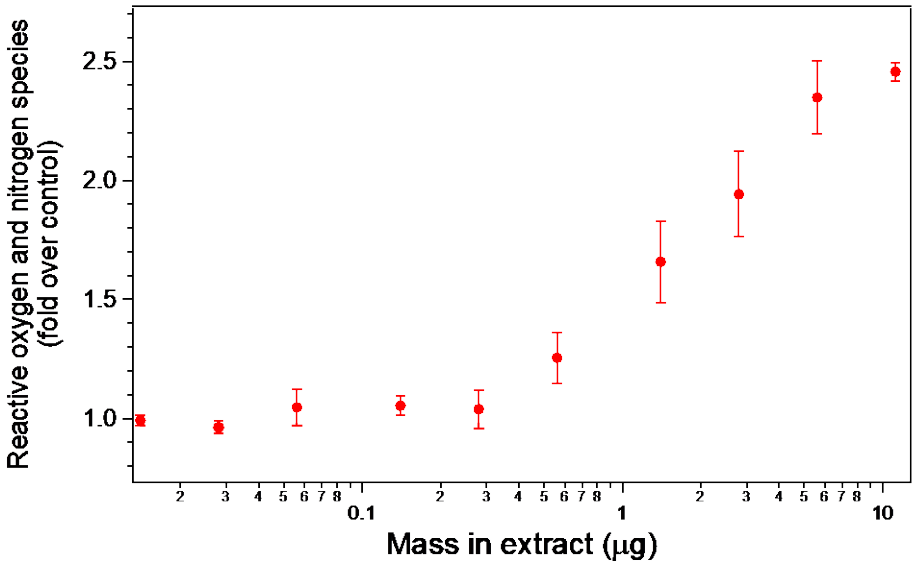
$$AUC_{extrinsic} = AUC_{intrinsic}\ x\ PM\ concentration$$

$$AUC_{extrinsic} = \frac{3}{\mu g}\ x\ 20\ \frac{\mu g}{m^3} = \frac{60}{m^3}$$

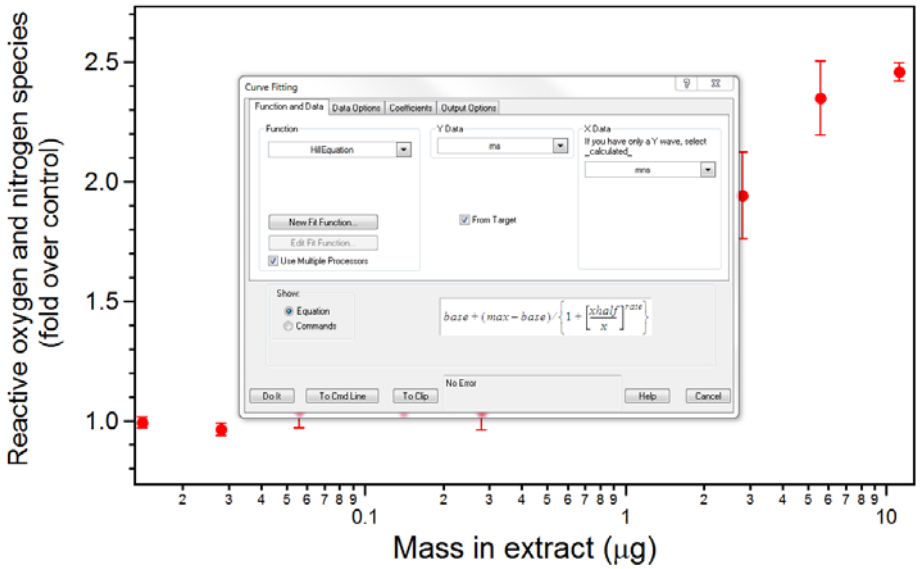
**AUC determination:**

*The following figures show how the AUC was obtained using the Hill equation fit and mathematically computing the area under the fit.*

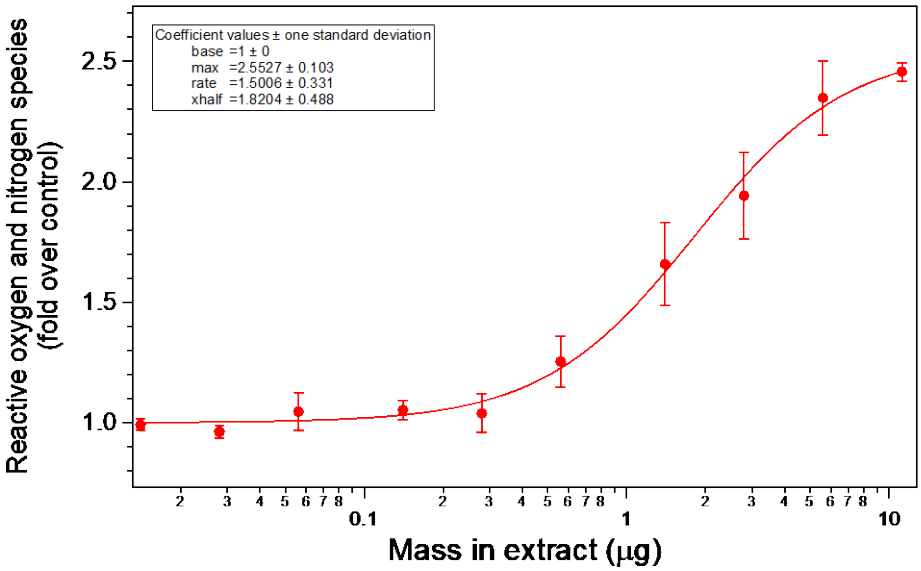
*Raw data (Expt 12). This example shows the case for when the maximum response is attained:*




*Choose Hill equation fit in Igor:*

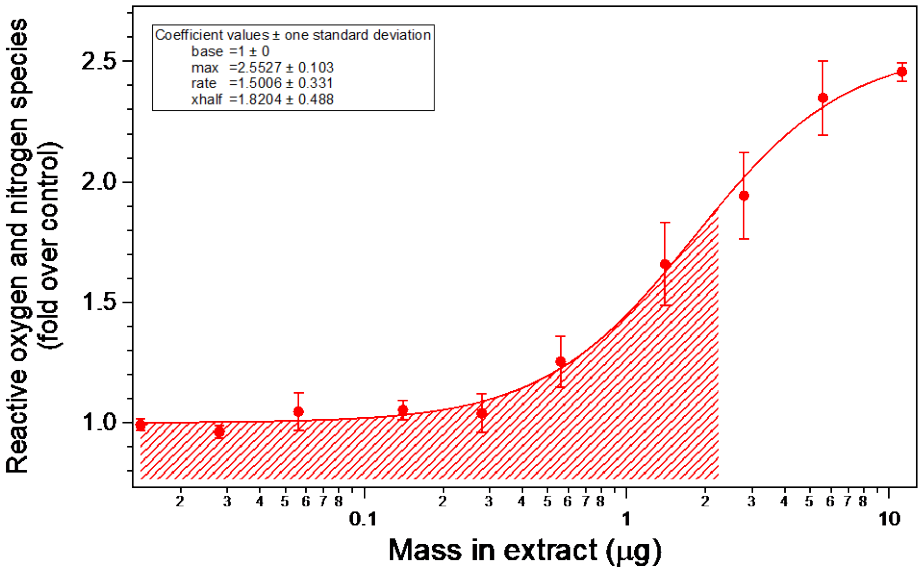


*Results from fit:*



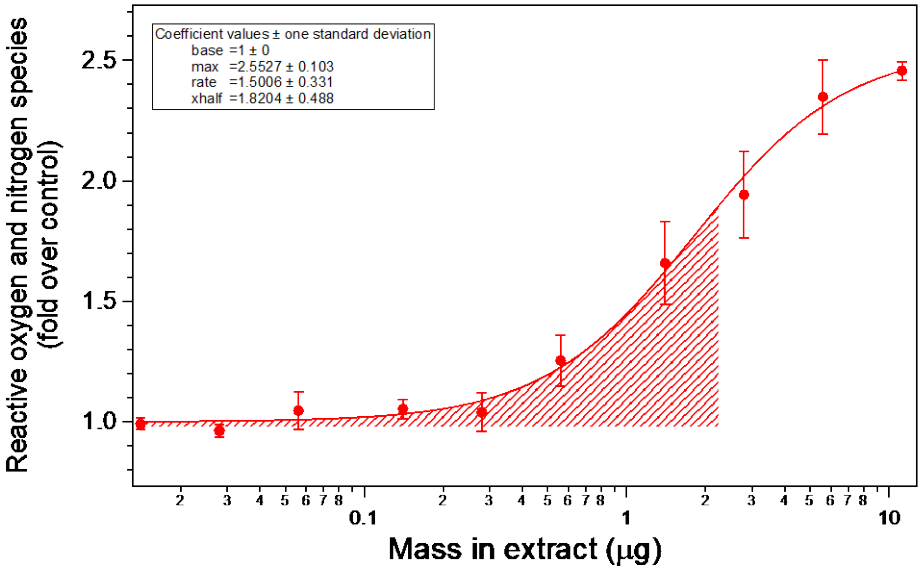

*Mathematically calculate area under fit curve:*

•print AreaXY(fitX_rns, fit_rns,0,2.27866348243299)
3.37821



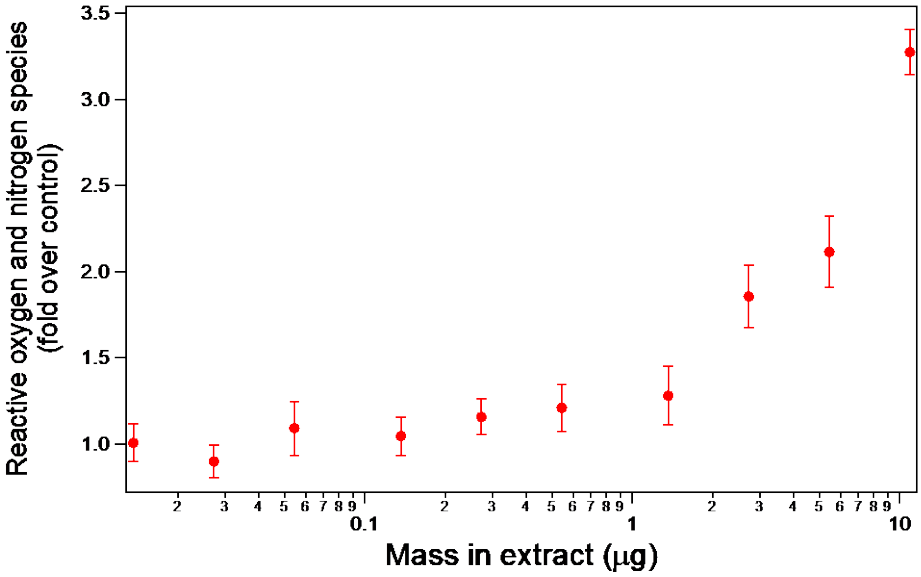
*Subtract area under baseline (fold over control = 1):*

```
•print AreaXY(fitX_rns, fit_rns,0,2.27866348243299) - 2.27866348243299
 1.09955
```

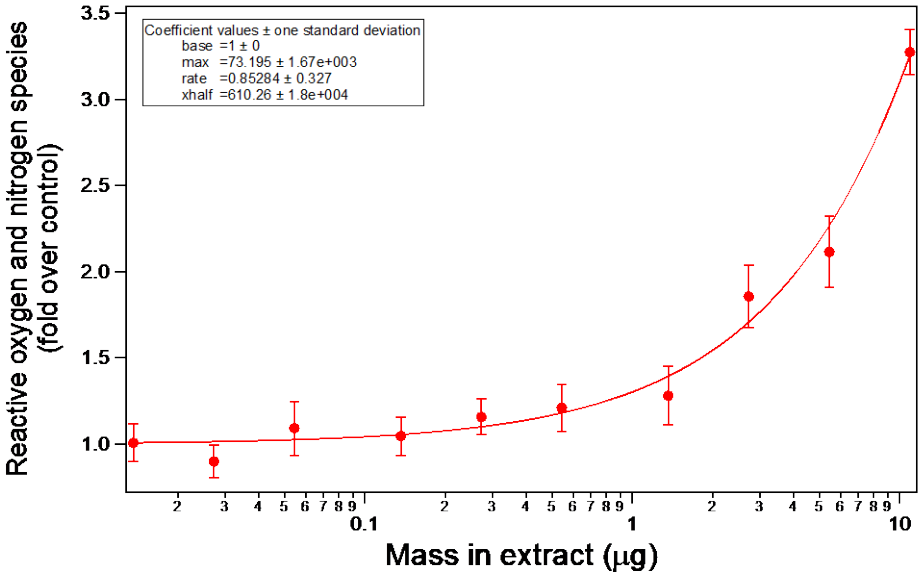



*This value (1.09955) is the calculated AUC for this experiment.*

*Raw data (Expt 18). This example shows the case for when the maximum response is not attained:*

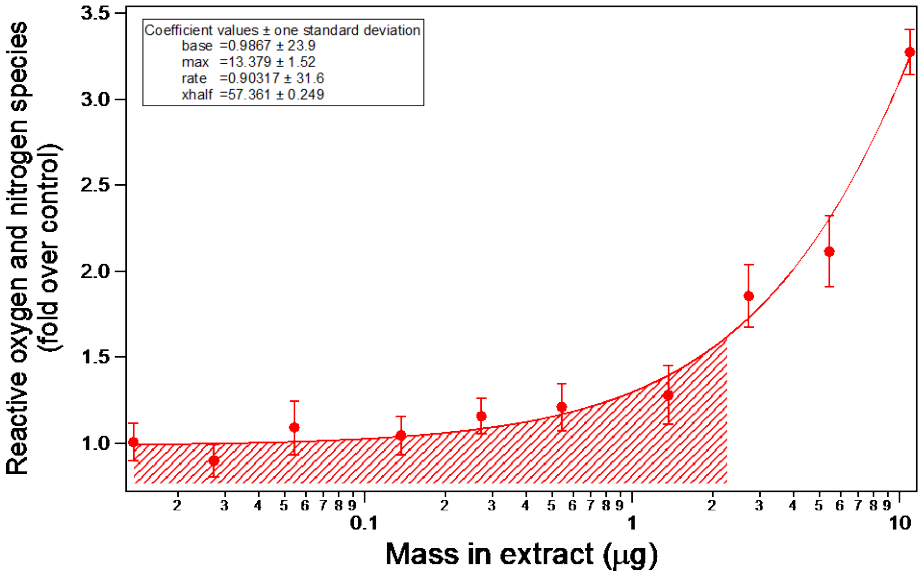



*Choose Hill equation fit in Igor and results from fit:*



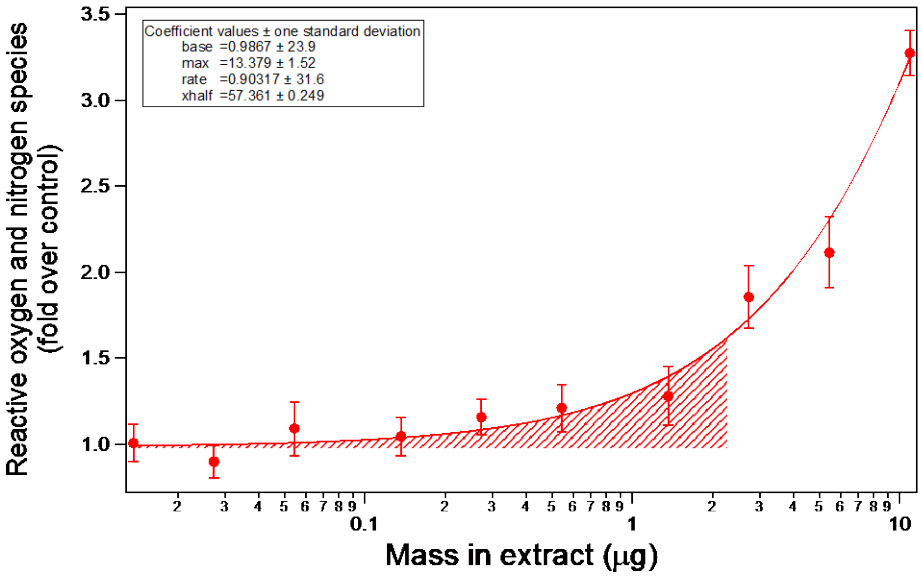
*Mathematically calculate area under fit curve:*

```
•print AreaXY(fitX_rnn, fit_rnn, 0, 2.27866348243299)
 3.01185
```




*Subtract area under baseline (fold over control = 1):*

```
•print AreaXY(fitX_rnn, fit_rnn, 0, 2.27866348243299) - 2.27866348243299
 0.733189
```




*This value (0.7332) is the calculated AUC for this experiment.*