
Presentation

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The Mechanisms of Arsenic bioremediation from water by the Green Microalgae *Chlorella vulgaris*

Leonardo Pantoja, Diane Purchase, Huw Jones, Jörg Feldmann and Hemda Garellick
Toxicity of arsenic to *C. vulgaris*
- ASTM E1218 and flow cytometry

Focused sonication for extraction for As-phytochelatin complexes

Formation of As-GS/PC complexes as detoxification mechanism
- Exposure to As(III) (Sodium arsenite)
- Exposure to DMA(V) (Dimethylarsinic acid)
- Exposure to As(V) (Sodium arsenate)

Total As-GS/PC formation

Transport of As-GS/PC to vacuoles

Conclusions
The Elements According to Relative Abundance

A Periodic Chart by Prof. Wm. F. Sheehan, University of Santa Clara, CA 95053

Colors suggest relative electronegativity

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Arsenic toxicity (similarities)
Arsenic toxicity (similarities)

![Graph showing the pH of phosphate and arsenate forms](image)

- $\text{H}_3\text{PO}_4$
- $\text{H}_3\text{AsO}_4$
- $\text{H}_2\text{PO}_4^-$
- $\text{H}_2\text{AsO}_4^-$
- $\text{HPO}_4^{2-}$
- $\text{HAsO}_4^{2-}$
- $\text{PO}_4^{3-}$
- $\text{AsO}_4^{3-}$
Arsenic toxicity (similarities)
TOXICITY OF ARSENIC TO *C. VULGARIS*

- Toxicity of As(III) (72 h @ 0.3mg L\(^{-1}\) phosphate) 
  \[ IC_{50} = 64.23 \text{ mg L}^{-1} \]
- Toxicity of As(V) (72 h @ 0.3mg L\(^{-1}\) phosphate) 
  \[ IC_{50} = 1.07 \text{ mg L}^{-1} \]

Chlorophyll as surrogate for cell health

### Toxicity of As(III) (72 h @ 0.3mg L\(^{-1}\) phosphate)

- **CMFDA**
  - Non protein SH reporter dye
  - D=0.03

- **BCECF**
  - pH reporter dye
  - D=0.06

### Toxicity of As(V) (72 h @ 0.3mg L\(^{-1}\) phosphate)

- **CMFDA**
  - Non protein SH reporter dye
  - D=0.56

- **BCECF**
  - pH reporter dye
  - D=0.32

### As(III) and As(V) Comparison

- **H2DCHF**
  - H-DA radical reporter dye
  - D=0.02

- **HE**
  - O\(_2\) superoxide radical reporter dye
  - D=0.03

**Kolmogorov-Smirnov statistics**

- n=20,000 cells, Strong D>0.3, Moderate 0.2< D <= 0.3, Weak 0.15< D <=0.20, Negligible D <= 0.15
ENHANCED DETERMINATION OF ARSENIC–PHYTOCHELATIN COMPLEXES IN *C. VULGARIS* USING FOCUSED SONICATION EXTRACTION*

1% formic acid, 30s in ice bath (4°C) @13mg L⁻¹ phosphate

<table>
<thead>
<tr>
<th>Sample</th>
<th>µg As g⁻¹</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total extraction <em>C. vulgaris</em></td>
<td>83.2</td>
<td>SE = 2.04, n= 6</td>
</tr>
<tr>
<td>Sonication <em>C. vulgaris</em></td>
<td>59.2</td>
<td>SE = 1.14, n = 6</td>
</tr>
</tbody>
</table>

% Recovery

<table>
<thead>
<tr>
<th>Quality control</th>
<th>µg As L⁻¹</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total extraction Kelp</td>
<td>23.5</td>
<td>SE = 0.60, n = 9</td>
</tr>
<tr>
<td>Sonication Kelp</td>
<td>22.2</td>
<td>SE = 0.28, n = 11</td>
</tr>
<tr>
<td>SRM 2669</td>
<td>48.1</td>
<td>%RSD = 2.16</td>
</tr>
<tr>
<td>Certified value</td>
<td>50.7</td>
<td>± 6.3 (95% CI)</td>
</tr>
</tbody>
</table>

SE= Standard error, n = Number of samples, % RSD = Relative standard deviation

# EXPOSURE TO As(III)*

Cells exposed to 50mg L⁻¹ for 48h

## Unbound peptides

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Formula</th>
<th>Monoisotopic mass (M+H⁺ or M+2H⁺)</th>
<th>Experimental mass</th>
<th>Difference ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH/PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>C₁₀H₁₇N₃O₆S</td>
<td>308.0916</td>
<td>308.0912</td>
<td>-1.34</td>
</tr>
<tr>
<td>GSSG</td>
<td>C₂₀H₃₂N₈O₁₂S₂</td>
<td>613.1598</td>
<td>613.1598</td>
<td>0</td>
</tr>
<tr>
<td>Reduced PC₂</td>
<td>C₁₈H₂₉N₅O₁₀S₂</td>
<td>540.1434</td>
<td>540.1433</td>
<td>-0.16</td>
</tr>
<tr>
<td>Oxidised PC₂</td>
<td>C₁₈H₂₇N₅O₁₀S₂</td>
<td>538.1278</td>
<td>538.1289</td>
<td>2.04</td>
</tr>
<tr>
<td>Reduced PC₃</td>
<td>C₂₆H₴₁N₁₇O₁₄S₃</td>
<td>772.1952</td>
<td>772.1952</td>
<td>-0.01</td>
</tr>
<tr>
<td>Oxidised PC₃</td>
<td>C₂₆H₴₉N₁₇O₁₄S₃</td>
<td>770.1795</td>
<td>770.1795</td>
<td>0</td>
</tr>
<tr>
<td>Reduced PC₄</td>
<td>C₃₄H₄₅N₉O₁₈S₄</td>
<td>1004.247</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala GSH/PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-(Glu-Cys)-Ala</td>
<td>C₁₁H₁₉N₂O₅S</td>
<td>322.1073</td>
<td>322.1072</td>
<td>-0.21</td>
</tr>
<tr>
<td>γ-(Glu-Cys)₂-Ala</td>
<td>C₁₉H₃₁N₈O₁₀S₂</td>
<td>554.1591</td>
<td>554.1578</td>
<td>-2.2</td>
</tr>
<tr>
<td>desGly GSH/PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-(Glu-Cys)</td>
<td>C₈H₁₄N₂O₃S</td>
<td>251.0702</td>
<td>251.0706</td>
<td>1.55</td>
</tr>
<tr>
<td>γ-(Glu-Cys)₂</td>
<td>C₁₈H₂₆N₄O₅S₂</td>
<td>483.1219</td>
<td>483.1217</td>
<td>-0.58</td>
</tr>
<tr>
<td>γ-(Glu-Cys)₃</td>
<td>C₂₄H₳₈N₆O₁₃S₃</td>
<td>715.1737</td>
<td>715.1747</td>
<td>1.41</td>
</tr>
</tbody>
</table>

## Arsenic bound peptides

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Formula</th>
<th>Monoisotopic mass (M+H⁺ or M+2H⁺)</th>
<th>Experimental mass</th>
<th>Difference ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH/PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As(III)-PC₂</td>
<td>C₁₈H₂₈N₉O₁₁S₂As</td>
<td>630.0443</td>
<td>630.0437</td>
<td>-0.88</td>
</tr>
<tr>
<td>As(III)-PC₃</td>
<td>C₂₆H₃₈N₁₄O₁₄S₃As</td>
<td>844.0933</td>
<td>844.0931</td>
<td>-0.19</td>
</tr>
<tr>
<td>GS-As(III)-PC₂</td>
<td>C₂₉H₄₃N₆O₁₈S₃As</td>
<td>460.0666</td>
<td>460.0663</td>
<td>-0.06</td>
</tr>
<tr>
<td>As(III)-(PC₃)₂</td>
<td>C₃₉H₅₄N₁₀O₂₅S₄As</td>
<td>576.0925</td>
<td>576.0923</td>
<td>-0.37</td>
</tr>
<tr>
<td>As(III)-PC₄</td>
<td>C₃₄H₅₀N₉O₁₈S₄As</td>
<td>1076.1451</td>
<td>1076.1455</td>
<td>0.37</td>
</tr>
<tr>
<td>MMA(III)-PC₂</td>
<td>C₁₈H₃₀N₅O₁₀S₂As</td>
<td>628.0728</td>
<td>628.0729</td>
<td>0.12</td>
</tr>
<tr>
<td>Ala GSH/PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As(III)-γ-(Glu-Cys)₃-Ala</td>
<td>C₂₉H₄₀N₉O₁₄S₂As</td>
<td>858.109</td>
<td>858.1082</td>
<td>-0.87</td>
</tr>
<tr>
<td>GS-As(III)-γ-(Glu-Cys)₂-Ala</td>
<td>C₂₉H₃₈N₈O₁₆S₂As</td>
<td>467.0744</td>
<td>467.0744</td>
<td>0.09</td>
</tr>
<tr>
<td>As(III)-γ-(Glu-Cys)₂-Ala</td>
<td>C₃₉H₅₇N₁₀O₂₀S₃As</td>
<td>583.1003</td>
<td>583.101</td>
<td>1.19</td>
</tr>
<tr>
<td>MMA(III)-γ-(Glu-Cys)₂-Ala</td>
<td>C₂₀H₃₂N₆O₁₀S₂As</td>
<td>642.0885</td>
<td>642.0889</td>
<td>0.66</td>
</tr>
</tbody>
</table>

## Newly reported peptides

Glutathione/PC homologues (terminal amino acid Gly substituted by Ala, Ser, Gln, Glu or is absent)

Newly reported peptides

EXPOSURE TO DMA*

Cells exposed to 50mg L^{-1} for 48h

DMAS^{V}-GS has only been reported once in Brassica Oleracea plants:

- Cabbage, broccoli, cauliflower, kale, Brussels sprouts, collard greens, savoy, among other

1 GSH (308), 2 DMAS^{V}-GS (444), 3 Reduced PC_{2} (540) and 4 Oxidised PC_{2} (538), As and S signals from HR-ICP-MS

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Formula (M)</th>
<th>Monoisotopic mass (M+H^+)</th>
<th>Experimental mass</th>
<th>Difference ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>C_{10}H_{17}N_{3}O_{6}S</td>
<td>308.0916</td>
<td>308.0918</td>
<td>0.65</td>
</tr>
<tr>
<td>Red PC_{2}</td>
<td>C_{18}H_{29}N_{5}O_{10}S_{2}</td>
<td>540.1434</td>
<td>540.1433</td>
<td>-0.16</td>
</tr>
<tr>
<td>Ox PC_{2}</td>
<td>C_{18}H_{28}N_{5}O_{10}S_{2}</td>
<td>538.1278</td>
<td>538.1288</td>
<td>1.86</td>
</tr>
<tr>
<td>DMAS^{V}-GS</td>
<td>C_{12}H_{23}N_{3}O_{6}S_{2}As</td>
<td>444.0244</td>
<td>444.0247</td>
<td>0.56</td>
</tr>
</tbody>
</table>

@13mg L^{-1} phosphate

EXPOSURE TO As(V)*

Cells exposed to 50mg L⁻¹ for 48h

@13mg L⁻¹ phosphate

At this level of phosphate cells are not under stress

Anal. Methods, 2014, 6, 791-797
EXPOSURE TO As(III)*

@13mg L⁻¹ phosphate
TOTAL As-GS/PC FORMATION

@13mg L\(^{-1}\) phosphate

- Coloured bars - total amount of arsenic
- White bars - amount of As-GS/PC (percentage of total arsenic)

Vertical bars denote + 1 standard error, n = 3
Toxicity (72 h) to As(III) (50 mg L\(^{-1}\)) @13mg L\(^{-1}\) phosphate

Test the presence of ABCC1 and ABCC2 inhibitors:

- MK571 (25 \(\mu\)M)
- Probenecid (Prob, 500 \(\mu\)M)
- Sodium taurocholate (Tau, 50 \(\mu\)M).

ABC = ATP-binding cassette transporter  
MRP = Multidrug resistance-associated protein

* (P < 0.05) with respect to control  
+ (P < 0.05) with respect to absence/presence of arsenic  
Vertical bars + 1 standard error  
Control n=6, experiments n = 3
TRANSPORT OF As-GS/PC TO VACUOLES

Cells exposed to 150 mg L\(^{-1}\) of As(III) for 24 h

**Without inhibitor**
- ABCC transport
- No transport

**With inhibitor**

**CMFDA substrate for ABCC transport**
- Probenecid
- MK571

**Treatment time 60 min, n=20,000 cells**

**Graphs and Data**
- Control - Probenecid
  - \(x = 52.9, \ SE = 3.2, n = 12\)

- Control - MK571
  - \(x = 59.2, \ SE = 2.9, n = 12\)

- Probenecid
  - \(x = 52.9, \ SE = 3.2, n = 12\)

- MK571
  - \(x = 59.2, \ SE = 2.9, n = 12\)

**Statistics**
- \(x = 25.9, \ SE = 8.2, n = 10\)
  - \(* p=0.005\)

**Treatment**
- \(D=0.08\)
- \(D=0.28\)

**Legend**
- Strong \(D>0.3\), Moderate \(0.2 < D <= 0.3\), Weak \(0.15 < D <=0.20\), Negligible \(D <= 0.15\)

**Notes**
- Cells exposed to 150 mg L\(^{-1}\) of As(III) for 24 h
- CMFDA substrate for ABCC transport
- Probenecid
- MK571

**Additional Information**
- Treatment time 60 min, n=20,000 cells
CONCLUSIONS

• As(V) is more toxic than As(III) to *C. vulgaris* cells at the same concentration of phosphate
• As(III) triggers the formation of **As-GS/PC** molecules
• As(V) does not trigger the formation of **As-GS/PC** molecules when cells are not under stress
• DMA triggers the formation of **DMAS^v-GS** but it is unlikely that this is part of a detoxification mechanism
• ABCC1 and ABCC2 are involved in **As-GS/PC** transport to acidic vacuoles in *C. vulgaris*
Thank you for listening.

Any questions?
**U1-3** Unknowns

**P4** GS-As(III)-PC$_2$/GS-As(III)-γ-(Glu-Cys)$_2$,

**P5** As(III)-γ-(Glu-Cys)$_2$

**P6** GS-As(III)-PC$_2$

**P7** GS-As(III)-γ-(Glu-Cys)$_2$-Ala

**P8** As(III)-PC$_3$/MMA(III)-PC$_2$

**P9** MMA(III)-PC$_2$

**P10** As(III)-PC$_3$/As(III)-(PC$_2$)$_2$

**P11** As(III)-(PC$_2$)$_2$/As(III)-γ-(Glu-Cys)$_3$-Ala/As(III)-γ-(Glu-Cys)$_2$-Ala/MMA(III)-γ-(Glu-Cys)$_2$-Ala,

**P12** As(III)-PC$_4$

**P13** As(III)-PC$_4$