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# Sub-lethal cadmium exposure increases phytochelatin concentrations in a mollusc

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## Abstract

Phytochelatin is a metal-binding metabolite found in almost all plant species and some animal groups, including nematodes and annelids, where they can play an important role in detoxifying metals such as cadmium. Species from several other taxa contain a phytochelatin synthase (PCS) gene orthologue, including molluscs, indicating they may have the potential to synthesize phytochelatin. However, the presence of a gene alone does not demonstrate that it plays a functional role in metal detoxification. In the present study, we show that the aquatic snail *Lymnaea stagnalis* produced both penta- and heptapeptide phytochelatin (i.e. phytochelatin-2 and phytochelatin-3), and their levels increased in response to sub-lethal levels of cadmium.

## Highlights

- We detected phytochelatin (PC<sub>2</sub> and PC<sub>3</sub>) in a mollusc species, *Lymnaea stagnalis*
- Phytochelatin increased in *Lymnaea stagnalis* when exposed to cadmium
- Future research on phytochelatin responses in molluscs would be valuable

## Keywords

*Lymnaea stagnalis*, cadmium, metal pollution, phytochelatin, detoxification

## Introduction

Phytochelatins (PCs) are non-ribosomal peptides, that are produced from glutathione by the action of a phytochelatin synthase (PCS) enzyme (Cobbett and Goldsbrough, 2002). They have the structure  $(\gamma\text{-GluCys})_n\text{Gly}$ , with  $n$  so far observed to range from 2 – 4 for animal species. The free thiols on the cysteine residues can strongly chelate soft metals and metalloids, such as cadmium and arsenic. PCs were first discovered in fission yeast and in plants, but were furthermore shown to be present and functional in an animal species: the nematode model organism *Caenorhabditis elegans*, where knocking down the PCS enzyme made them hypersensitive to cadmium (Clemens et al., 2001; Vatamaniuk et al., 2001). PCS mutants also have an increased accumulation of cadmium compared to wild-type, indicating that PCS plays a role in metal detoxification (Essig et al., 2016).

Metallothioneins (MTs) are small proteins generally thought of as the prototypical thiol-rich metal-binding molecules in animals, and there are many studies on their responses and role in animal species (Amiard et al., 2006). MTs may not necessarily be the main players in detoxification, though, as knocking out the PCS gene in *C. elegans* makes it more sensitive to cadmium than does knocking out the two MT genes (Hughes et al., 2009). This has obvious and direct relevance for ecotoxicological research: perhaps PCs will turn out to be of major importance for many environmentally-relevant animal species. Studies on metal pollution which have not analysed PCs may turn out to give an incomplete picture (Bundy et al., 2014). Indeed, recent studies have shown direct responses of PCs to metal(loid) ions in animal species from additional phyla, including earthworms (Annelida) and sea squirts (Chordata) (Franchi et al., 2014; Liebeke et al., 2013). This, again, highlights the potential significance of PCs in the context of environmental pollution research, as they are found in phyla with members from the terrestrial, freshwater, and marine environments.

This still leaves one of the most ecologically relevant and speciose phyla unaccounted for – what about molluscs? Molluscs are also found in all three major environments (terrestrial, freshwater, and marine), and include key sentinel and monitoring species – many shellfish are of particular ecological and economic interest because they are filter-feeders and so can accumulate high levels of pollutants, and may be part of the human food chain. At least some mollusc species contain PCS genes, including gastropod and bivalve species (Bundy and Kille,

2014). Indeed PCS gene transcription was affected in response to metal ions in the mussel *Bathymodiolus azoricus* (Bougerol et al., 2015). However, gene expression is probably not a reliable reporter of actual changes in the functional end products, the PCs (Essig et al., 2016; Liebeke et al., 2013). In the present study we exposed a mollusc species, the freshwater snail *Lymnaea stagnalis*, to sub-lethal levels of cadmium (Cd), and used direct biochemical detection of PCs to determine the snails' response.

## Materials and Methods

### Culture and metal exposures

*Lymnaea stagnalis* cultures were established from a parasite-free culture established at the INRA centre (Rennes, France). The snails were acclimated (phase 1) in aquaria with tap water for two weeks and maintained under controlled conditions: light/dark photoperiod cycle of 16/8 hours; temperature of  $20 \pm 1^\circ\text{C}$ ; pH of  $7.5 \pm 0.5$ ; oxygen concentration  $> 6 \text{ mg L}^{-1}$ ; and ammonium concentration  $< 1.5 \text{ mg L}^{-1}$ . One-third of the water was renewed weekly, and the snails were fed *ad libitum* with organic lettuce three times a week. The snails were then transferred into 1L glass vials for acclimation to the test conditions (phase 2, chronic exposure) until reproduction restarted. For phase 2, five concentrations of Cd (25, 50, 100, 200 and  $400 \mu\text{g Cd L}^{-1}$ ) were used, with six replicates per concentration and five snails (shell length  $2.7\text{cm} \pm 0.2$ ) per replicate, plus a negative control with water only. The snails were exposed for 56 days, under the same light/dark cycle and physical-chemical conditions as described for the cultures. Full medium renewals were made twice a week (semi-static test) and snails fed *ad libitum* with organic lettuce. Survival and fecundity were also recorded at least twice a week, and dead snails were withdrawn from the vials. Individual snails from each replicate vial were used for PC and Cd analysis.

### Metals analysis

After the 56-day exposure, snails were lyophilized for 48h and pooled by replicate within each treatment. Then, for each replicate, the pool was ground and aliquots of the powdered tissue weighed for acid digestion. The tissue was digested in a mixture of trace analysis grade nitric acid ( $\geq 69\%$ ) + perchloric acid (67-72%), in a proportion of 7:1 v/v (1 ml for samples  $< 1\text{mg}$  and 2 ml for samples  $\geq 1 \text{ mg}$ ), in a four-step temperature cycle ( $90^\circ\text{C}$ ,  $120^\circ\text{C}$ ,  $140^\circ\text{C}$ , and  $160^\circ\text{C}$ ) as described by Ardestani and van Gestel (2013). After cooling to room temperature, 10 ml of nitric acid (0.1M) was added to each digest.

ICP-MS analysis was carried out with an i-CAP Qc instrument (Thermo Scientific, UK) in He-mode (KED-mode). External standard solutions were prepared from 10 mg/kg multi element standard AccuTrace® (AccuStandard®, New Haven, USA). Germanium was obtained from High-Purity Standards, USA and diluted to 5 µg/L using 0.1 % (v/v) nitric acid. The instrument was optimised for highest sensitivity under robust plasma conditions (high matrix insert in skimmer), with Ni-cones and Micromist nebulizer. He was used at 4.5 mL/min as the reaction cell gas. Integration time per isotope was 0.1 s, with 5 replicates each. Internal standard (Ge, 5 µg/L) was continuously added via a T-piece before the nebulizer. Blank samples were included in the analysis process, as well as certified reference material DOLT-3 (Dogfish liver) from National Measurement Standard of Research Council of Canada. Cd was below detection limits in all of the blank samples, and the CRM samples had a mean value of 24.2 mg Cd kg<sup>-1</sup> (125% of the certified value), with a coefficient of variance of 35% (omitting one sample out of six for which no signal was obtained).

### PC analysis

We ground each whole snail (approximately 1g wet weight) into 3 ml of methanol (containing 9.8 mM tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent) using a pestle and mortar at liquid nitrogen temperatures, allowed the frozen powder to thaw, and transferred the extract to a microcentrifuge tube. Following centrifugation (5 min, 16,000g), the supernatants were derivatized with 60 µl of 266 mM *N*-ethyl maleimide. We then analysed the phytochelatins PC<sub>2</sub> and PC<sub>3</sub> using a targeted LC-MS-MS method, based on the approach of Liebeke et al. (2013). Briefly, samples were injected onto an Agilent 1200 HPLC system coupled to a SCIEX 6500 Q-TRAP mass spectrometer (AB SCIEX, Warrington, UK) operated in High Mass negative mode. Chromatography was performed using a C18 column (100 x 3 mm, Ascentis Express, Sigma-Aldrich, Poole, UK) and a gradient of 100% A (94.9% H<sub>2</sub>O; 5% acetonitrile; 0.1% formate) to 100% B (5% H<sub>2</sub>O; 94.9% acetonitrile; 0.1% formate) over 6 min. at 40°C. The solvent flow rate was 250µl/min. PC<sub>2</sub> and PC<sub>3</sub> had a retention time of 4.2 and 4.5 minutes, respectively. Data were acquired and analysed with Analyst 1.4.2 software (Applied Biosystems). We used four mass transitions to monitor each phytochelatin (Table 1). Collision cell exit potentials were set at -50v and -13v respectively.

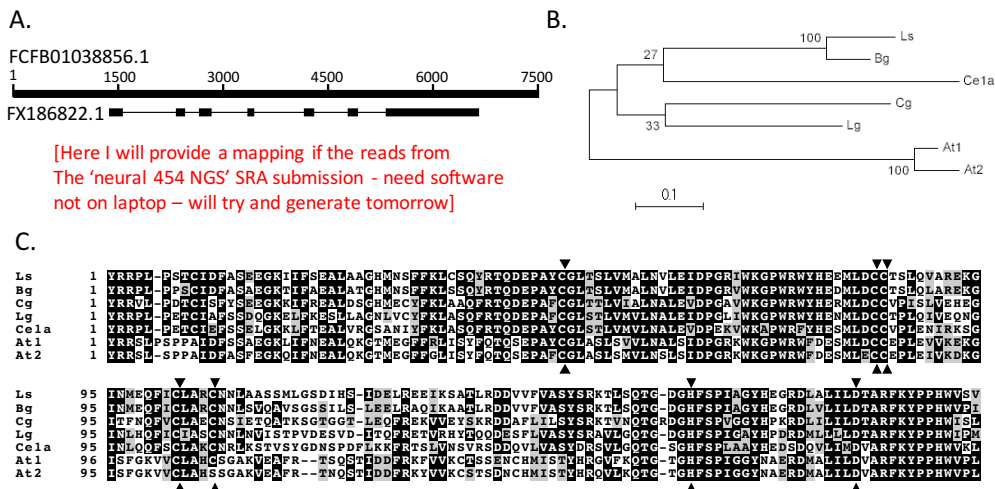
**Table 1.** Mass spectrometer parameters for detection of NEM-derivatized phytochelatins.

Q1 Mass (Da)	Q3 Mass (Da)	Dwell (ms)	Collision energy
790.2	661.4	50	30
790.2	358.3	50	45
790.2	304.3	50	45
790.2	267.3	50	70
1147.2	1018.4	50	45
1147.2	586.4	50	60
1147.2	558.4	50	70
1147.2	358.1	50	70

## Results and Discussion

Three out of the five cadmium concentrations were high enough to kill some or all of the snails during the exposure period: 100% of the snails died at the two highest concentrations (200 and 400 µg Cd l<sup>-1</sup>), and 90% died at the middle concentration (100 µg Cd l<sup>-1</sup>), with a calculated LC<sub>50</sub> value of 78.2 ± 1.5 (S.E.) µg Cd l<sup>-1</sup>. We therefore carried out all further analyses (metal and PCs) only for the snails exposed to sub-lethal cadmium concentrations (i.e. 0, 25 and 50 µg Cd L<sup>-1</sup>). Gomot (1998) reported low mortality during a 7-week exposure period, where 10 to 20% mortality was observed under similar Cd concentrations used in the present study. In addition sub-lethal effects on egg development were observed at low concentrations (25 µg l<sup>-1</sup>). Coeurdassier et al. (2003) were unable to obtain an LC<sub>50</sub> for *L. stagnalis* exposed to Cd at concentrations up to 320 µg L<sup>-1</sup>, but the period of exposure was shorter (28 days). Such time dependency of effects is consistent with the known slow toxicokinetics of Cd.

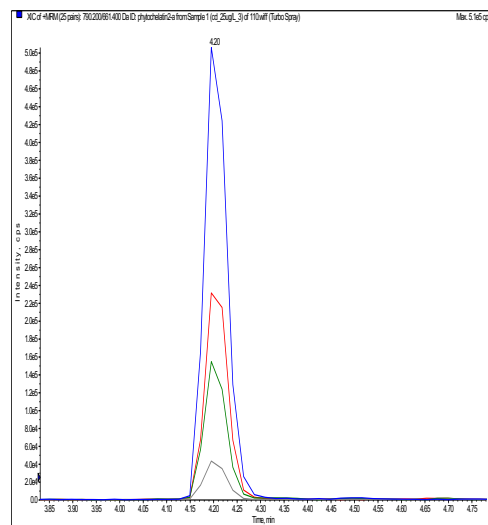
The exposure increased the snails' body burden of Cd. Tissue Cd concentrations were below detection limits for the control group, and the 25 and 50 µL Cd L<sup>-1</sup> exposure groups both had elevated Cd levels, 72 ± 11 mg/kg and 63 ± 15 mg/kg (mean ± SD, n=6) respectively. This suggested the possibility of analysing the data as a two-class study, i.e. considering both the nominal exposure levels to have effectively the same Cd level, since the two Cd exposure groups were not significantly different from each other (t test, P = 0.25).



**Figure 1.** Gene structure, organisation and evolutionary conservation of *Lymnaea stagnalis* phytochelatin synthase. Panel A provides an illustration of the complete loci encoding the *L. stagnalis* PCS gene, located on draft genome assembly gLs.1.0.scaf01115\_contig\_7 (Gb:FCFB01038856 join(1405..1572,2333..2438,2653..2825,336..3436,4159..4277,4759..4903,5293..6121)). Evidence for the transcript was provided by a full gene assembly from Transcript Shotgun assembly performed from *Lymnaea* CNS (FX186822: CDS(154..1866) (Sadamoto et al., 2012) together with significant mapped reads from SRA bio-projects representing transcripts from Embryo 32 and I cell embryos (SRA: ERX631987 & ERX631986). Panel B shows a maximum likelihood phylogenetic tree of the *L. stagnalis* PCS (*Ls* - FX186822) together with that predicted from an un-annotated transcript from the aquatic snail *Biomphalaria glabrata* mRNA (*Bg* - XM\_013214798.1); representatives of two additional Mollusca PCS, *Lottia gigantea* (*Lg*PCS - ESP03342 and *Crassostrea gigas* (*Cg*PCS - EKC27807); a PCS from the nematode *Caenorhabditis elegans* (*Ce1a* - A5JYS0); and two plant isoforms from *Arabidopsis thaliana* (*At1* - Q9S7Z3, *At2* - Q9ZWB7). Panel C shows an alignment of the PCS region used for the phylogenetic analysis with residues showing >50% conservation shown with a black background, and with those showing functional conservation shown in grey. Residues identified as essential for function are indicated by arrows.

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The *L. stagnalis* PCS gene has not been formally described. We obtained and annotated a sequence based on publically-available data (and have deposited the annotation to GenBank, accession number in process), which contains conserved key functional amino acids. A clustering analysis based on the conserved portion indicates that the *L. stagnalis* gene clusters together most closely with another snail PCS (*Biomphalaria glabrata*), and with a number of other animal PCS sequences (including two other mollusc species), away from plant PCS (Figure 1). The functionality of the *L. stagnalis* PCS enzyme was not directly demonstrated by expressing it in a suitable recombinant host, so it was important to check that changes in PCs were actually measured and not some other compound. We are confident that our analytical approach was specific for PCs, as four separate mass transitions were monitored for all samples (Figure 2).



**Figure 2.** Phytochelatin was detected specifically by LC-MS-MS, with co-elution of characteristic mass

transitions, and very low background signal: phytochelatin-2 signal in a sample extracted from an exposed snail.

PC<sub>2</sub> was present in all of the snail samples, including the controls, and PC<sub>3</sub> in all but one of the control samples, where it was below the detection limit. Both PCs had a highly non-normal distribution, but nonetheless there were

clear differences in response to Cd (Table 1). PC<sub>2</sub> was present at a concentration approximately an order of magnitude greater than PC<sub>3</sub>. Both increased in response to Cd, but although the relative concentrations of PC<sub>2</sub> remained higher than PC<sub>3</sub>, the Cd-induced increase in PC<sub>3</sub> was more statistically significant than for PC<sub>2</sub>. If the Cd-exposed samples were treated as a single group, then the increase in PC<sub>2</sub> + PC<sub>3</sub> was significant at  $P < 0.01$  (Table 2).

**Table 2.** Phytochelatin responses to Cd treatment (relative concentrations); median values are shown, with the data range in parentheses. P values were derived from one-tailed Mann-Whitney U tests for the control data vs. the 25 and 50  $\mu\text{g Cd l}^{-1}$  and the combined “Cd exposed” group (i.e. the 25 + 50  $\mu\text{g Cd l}^{-1}$  data combined).

Cd ( $\mu\text{g l}^{-1}$ )	PC <sub>2</sub>	PC <sub>3</sub>	PC <sub>2</sub> significance test	PC <sub>3</sub> significance test
0	5.82 (1.56-42.6)	0.435 (0-0.81)	-	-
25	119 (9.21-1010)	5.15 (0.84-147)	0.013	0.002
50	21.3 (11.5-585)	1.48 (0.77-108)	0.014	0.009
Cd exposed	60.2 (9.21-1010)	3.19 (0.77-147)	0.0045	0.001

Previous studies of *L. stagnalis* detoxification responses to Cd and other potentially toxic metals focussed on MTs, in both laboratory and field studies (Gnatyshyna et al., 2011; Leung et al., 2003; Ng et al., 2011). To our knowledge, no other study has directly analysed PC responses in any mollusc species. Our current study is a preliminary observation, since we have not yet fully demonstrated that PCs have a functional role in metal detoxification in *L. stagnalis*, although we do consider it is the most probable explanation. Of course, this kind of functional demonstration is much harder when working with non-model organisms like *L. stagnalis* compared to *C. elegans*, where it is relatively straightforward to obtain a mutant strain, or modulate gene activity by RNA interference. It is no coincidence that *C. elegans* is the animal species for which there is currently the greatest degree of knowledge about PC function. Nonetheless, one can still build up a mechanistic understanding of responses to toxins in non-model organisms, even though it may require a greater range and quantity of data (Bundy et al., 2008).

In our opinion, the growing body of evidence that PCs are metal-responsive in multiple animal species argues in favour of the potential importance of PC synthesis in invertebrate

responses to toxic metals (in at least some phyla). Phytochelatins are more protective than MTs in *C. elegans*, the sole animal species for which a direct comparison has yet been made (Hall et al., 2012; Hughes et al., 2009). Some patterns are starting to emerge: in the present study, PC<sub>2</sub> had much higher concentrations than PC<sub>3</sub>, but the increase in PC<sub>3</sub> was more statistically significant. Both of these observations are consistent with results from other animal species (earthworms and nematodes) (Hughes et al., 2009; Liebeke et al., 2013).

*L. stagnalis* contains a PCS orthologue, but the presence of a gene alone does not demonstrate that it plays a functional role in metal detoxification. It is not even sufficient to show that the gene product is potentially active. The PCS from the platyhelminth *Schistosoma japonicum* can synthesize PCs when heterologously expressed in fission yeast, but *S. japonicum* itself apparently does not produce PCs in response to Cd (Ray and Williams, 2011; Rigouin et al., 2013). Gene expression analysis adds another piece of evidence, but again may well be insufficient to show functionality in animal species. In plants, PC production is not regulated by transcription: the substrate for the PCS enzyme is glutathione coordinated by metal ions, so any entry of free metal ions into the cells

will result in formation of GSH complexes and rapid PC synthesis (Cobbett and Goldsbrough, 2002). There is much less information about the regulation of animal phytochelatin. Liebeke et al. (2013) found no association between PCS gene expression and PC production in response to arsenic in the earthworm *Lumbricus rubellus*. Similarly, low levels of Cd did not result in upregulated *pcs-1* gene expression in *C. elegans* (Cui et al., 2007). Other studies on different earthworm species had some intriguing hints about possible regulation, but because they did not measure PC levels directly, the relationship to gene expression could not be determined. Brulle et al. (2008) saw differences in PCS expression for *Eisenia fetida* at low but not high Cd concentrations, whereas Homa et al. (2015)

saw differences for *Eisenia andrei* for essential (Cu, Zn) but not non-essential metals (Cd, Pb).

In conclusion, our current study demonstrates that the *L. stagnalis* PCS gene is functional, as shown by synthesis of PCs in response to cadmium. A full understanding of the role of PCs in metal detoxification in *L. stagnalis* and in other molluscs will require considerable additional research. In particular, nothing is currently known about the kinetics of the PC response; the interaction (if any) of PCs with MTs; the role (if any) of gene transcription; and the responses to metals and metalloids other than Cd. Direct analysis of PCs will prove essential in future studies of animals with PCS genes.

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