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Rapid Sublethal Toxicity Assessment Using Bioluminescent *Caenorhabditis elegans*, a Novel Whole-Animal Metabolic Biosensor

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Sublethal metabolic effects are informative toxicological end points. We used a rapid quantitative metabolic end point, bioluminescence of firefly luciferase expressing *Caenorhabditis elegans*, to assess effects of sublethal chronic exposure (19 h) to the oxidative stress agent and environmental pollutant cadmium (provided as chloride salt). Bioluminescence declined in a concentration-dependent manner in the concentration range tested (0–30μM Cd), with comparable sensitivity to reproduction and developmental assay end points (after 67 and 72 h, respectively). Cd concentrations that resulted in 20% reduction in bioluminescence (EC_{20}) were 11.8–13.0μM, whereas the reproduction EC_{20} (67 h exposure) was 10.2μM. At low concentrations of Cd (≤ 15μM), the decline in bioluminescence reflected a drop in ATP levels. At Cd concentrations of 15–30μM, decreased bioluminescence was attributable both to effects of Cd on ATP levels and decreased production of luciferase proteins, concomitant with a decline in protein levels. We show that whole-animal bioluminescence is a valid toxicological end point and a rapid and sensitive predictor of effects of Cd exposure on development and reproduction. This provides a platform for high-throughput sublethal screening and will potentially contribute to reduction of testing in higher animals.

Key Words: Caenorhabditis elegans; **sublethal toxicity screening; ATP; firefly luciferase; cadmium.**

The challenge of assessing the environmental and public health impacts of human activities requires a comprehensive approach that integrates chemical analysis and biomonitoring. Complex mixtures of toxins are present in environmental situations, and prevailing field conditions will influence their bioavailability and biological impacts. The monitoring and understanding of these impacts have required increasingly sensitive sublethal bioassays using multiple organisms belonging to different levels of biological organization and functionality.

Energy-related end points are emerging as valuable parameters in sublethal ecotoxicity assessment. In diverse organisms, these end points have been measured as oxygen consumption (Cherkasov *et al*., 2006; Pistole *et al*., 2008; Rowe *et al*., 2001), activity of glycolysis enzymes (Almeida *et al*., 2001), and activity of Krebs cycle enzymes (Reddy and Bhagyalakshmi, 1994), etc. However, the use of these measurements in routine toxicity assessment is constrained by their labor-intensive nature.

Bacterial bioluminescence-based metabolic assays on the other hand have been successfully employed in ecotoxicological assessment and early warning of risk for some time as they offer a means for rapid, sensitive, and cost-effective toxicity testing (Paton *et al*., 2006; Shaw *et al*., 1999). They rely on natural or transgenic bioluminescence as a reporter of cellular respiratory reactions and, hence, metabolic status (Meighen and Dunlap, 1993; Prosser *et al*., 1996). We have extended this approach to the multicellular model nematode *Caenorhabditis elegans* (Hollis *et al*., 2001; Lagido *et al*., 2001, 2008) through the expression of the eukaryotic firefly luciferase gene. The usefulness of *C. elegans* in toxicology has been highlighted by several authors (Lagido, 2009; Leung *et al*., 2008) and extends beyond its taxonomical boundaries. Due to evolutionary conservation, mechanisms underlying toxicity to nematodes are likely to have parallels in other organisms, including humans.

In this study, we measure bioluminescence of luciferase expressing nematodes as a direct measure of the perturbation in ATP pools elicited by stress (Lagido *et al*., 2008). This is a conceptually different approach to other available *C. elegans* biosensors that report on the induction of specific stress responses under unfavorable environmental conditions, such as the induction of heat shock response or of metallothionein genes (Cioci *et al*., 2000; Link *et al*., 1999). The integration of multiple (sub)cellular and physiological effects of toxins leads to stress-related energy deficiency which is ultimately detrimental to the fitness and survival of the organism and therefore of high ecological relevance.

We have previously demonstrated in short (1–2 h) exposure assays that bioluminescence of firefly luciferase expressing *C. elegans* reported on the detrimental effect of copper, lead, 3-5 dichlorophenol, and thermal stress (Lagido *et al*., 2001). More recently, we have shown the connection between effective mitochondrial function and light production in these transgenic *C. elegans* strains (Lagido *et al*., 2008). As stress perturbs the cellular energy balance (Corton *et al*., 1994), we hypothesize that the *C. elegans* bioluminescence response to toxins is

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a useful sublethal end point in toxicology tests. To address this hypothesis, we tested the metabolic and bioluminescent response of *C. elegans* to longer exposures (19 h) of sublethal levels of the model environmental pollutant and oxidative stress agent, cadmium. This forms the basis for a novel, informative, chronic bioluminescence assay for monitoring the sublethal stress effects. This assay has comparable sensitivity to reproduction and developmental tests and permits faster quantification, as well as providing an insight into the physiological processes taking place upon toxic exposure. Bioluminescence introduces a powerful means for automation of sublethal toxicity screening and assessment of physiological status in the model nematode *C. elegans*, and therefore, it is likely to contribute to reduction of testing in higher animals.

MATERIALS AND METHODS

Caenorhabditis elegans strains and culture conditions. The strains used were N2 (wild type, Bristol) and the bioluminescent strains PE255, PE254, and PE322 (the parental strain from which PE255 and PE254 were derived) (Lagido *et al*., 2008). Nematodes were maintained on NGM agar plates, seeded with *Escherichia coli* OP50 as food, at 20°C (Brenner, 1974). *Caenorhabditis elegans* was grown in liquid medium by washing worms from one NGM plate into 30 ml K medium (52mM NaCl and 32mM KCl in ddH_2O) (Williams and Dusenbery, 1990) supplemented with cholesterol (5 μg/ml) and *E. coli* OP50 (15–30 g/l) and incubation at 160 revolutions per minute (rpm), 20°C. Synchronization of nematode cultures was achieved by bleaching gravid hermaphrodites and overnight hatching in M9 (Lewis and Fleming, 1995).

Cd standards. A top standard of 10mM Cd in ddH_2O was prepared from $CdCl_2$ (Fisher Scientific, C/0280/48), acidified with 0.1% (vol/vol) 1 N nitric acid, and stored at 4°C in the dark. A 1mM Cd standard was prepared freshly each time by dilution of the top standard into K medium plus cholesterol (5 μg/ml), pH adjusted to 5.5, and diluted further into K medium, pH 5.5, to prepare the working Cd standards used in exposure experiments. All Cd concentrations referred to are nominal concentrations.

Cd exposure prior to bioluminescence, fluorescence, in vitro luciferase, and in vitro ATP measurements. Synchronized L1 nematodes were washed twice by centrifugation at 600 × g, and nematodes (1×10^3 to 3×10^3/ml depending on the experiment) in a total volume of 0.9 ml were added to 12-well plates containing K medium plus cholesterol (5 μg/ml) and *E. coli* OP50 (15 mg/ml), both final concentrations. Worms were incubated (20°C, 160 rpm) for 36 h (late L3 stage) prior to adding 100 μl of Cd standards or control (K medium) to final concentrations of 0, 10, 15, 20, 25, and 30μM, bringing the total volume per well up to 1 ml. Each Cd treatment was replicated three or four times. Exposure to Cd was for 19 h prior to determination of bioluminescence, fluorescence, or harvesting of worms for *in vitro* luciferase assay as described below. This provided a relatively long exposure to Cd, but this exposure time did not incur progeny production. To assess nematode feeding during experiments, the optical density of the supernatants were determined following removal of the nematodes by a short centrifugation step (200 × g, 2 min).

Bioluminescence, fluorescence, and in vitro luciferase activity measurements. Bioluminescence was measured in relative light units and expressed as a percentage of controls. In experiments with the strain PE322, bioluminescence was measured after resuspension of harvested worms and 5-min incubation in luminescence buffer (citrate phosphate buffer, pH 6.5, plus 1% DMSO, 0.05% Triton X-100, and 100μM D-luciferin) as described in Lagido *et al*. (2001). In experiments with the strains PE254 and PE255, worm suspensions in the respective test solutions were placed in eight-well reagent reservoir (Labsystems, Finland) to enable transfer to standard opaque white 96-well plates (Nunc). Nematodes were maintained in suspension by shaking (160 rpm, IKA KS 130 B benchtop shaker; GMBH & Co.). Each test sample was replicated into six different wells (50 μl per well). Bioluminescence was read in a Clarity Luminometer (Biotek) for 1 s, after injection of, and 5-min incubation with 50 μl of 2× concentrated luminescence buffer.

For fluorescence readings and *in vitro* luciferase assays, worms were centrifuged (600 × g, 4°C, 1min) in 15-ml tubes (Cellstar, Greiner), washed 2× in ice-cold S basal + Tween 20, and concentrated to approximately 4×10^3/ml in the same medium. Triplicate aliquots (50 μl) of this suspension were placed in a Black Dinex microfluor 96-well plate. Fluorescence was quantified as described previously (Lagido *et al*., 2008).

To determine the activity of luciferase *in vitro*, the suspension of worms (~4×10^3/ml) was concentrated a further five times by allowing worms to settle to the bottom of the tube, which was kept on ice, and collecting a 100-μl pellet (in S basal + 0.01% Tween 20). Samples were fast frozen in liquid nitrogen, kept at −80°C for subsequent analysis of luciferase activity and protein levels as described previously (Lagido *et al*., 2008).

Assessment of development in the presence of Cd. Experiments were carried out in 12-well plates. Synchronized L1 nematodes (strain PE322) suspended in K medium plus cholesterol (5 μg/ml) and *E. coli* OP50 (30 mg/ml) were added to each well and mixed with an equal volume of a 2× concentrated Cd standard (total volume 1 ml). Nominal concentrations of Cd tested were 0, 10, 20, 30, and 100μM Cd, and experiments were carried out in triplicate. After an arbitrarily selected incubation of 72 h (20°C, 160 rpm), triplicate samples (10 μl) from each well were scored for developmental stage by observation of size and morphology (using brightfield microscopy and when required 200× magnification to observe development of the gonad and vulva) following paralysis of worms with a saturated suspension of phenoxypropanol (5% in M9). Strain PE322 contains both *luc::gfp* marked and unmarked nematodes. Fluorescence under epifluorescence optics was used to determine which nematodes carried the *luc::gfp* transgene, and only these were assessed so that bioluminescence and developmental data were comparable. Second-generation L1 larvae were not scored.

Assessment of reproductive output in the presence of Cd. Experiments were set up as described under "Cd Exposure Prior to Bioluminescence, Fluorescence, In Vitro Luciferase, and In Vitro ATP Measurements" section with approximately 1×10^3 worms/ml but exposure to Cd was for 67 h prior to assessment of effects on reproduction. These experimental conditions support the development of the first generation of the bioluminescence strains. An exposure time of 72 h is commonly used in reproduction experiments (Anderson *et al*., 2001; Traunspurger *et al*., 1997); however, the slightly shorter exposure time (67 h) was selected for experimental convenience. Each Cd concentration was replicated three times. Under all concentrations tested, all worms reached the adult stage, and after 67-h exposure, mixed populations of adults and progeny L1 larvae were present. Triplicate samples (10 μl) from each well were scored under microscopic observation for number of adults and of L1 larvae. In samples with high larvae numbers, scoring was carried out in 20× diluted samples. Lethality was less than 2% in all tested conditions.

Determination of in vitro ATP and protein levels. Following exposure to Cd, worms were collected as described for fluorescence measurements, and a 100-μl pellet of worms (in S basal plus 0.01% Tween 20) was snap frozen in liquid nitrogen and stored at −80°C until subsequent analysis. To control for any bacteria present in pellets following harvesting of worms, bacteria-only controls (15 mg/ml *E. coli* OP50) were included in experiments. Bacterial contribution toward the *in vitro* ATP levels and total protein was negligible. Worm extracts were prepared by disruption with glass beads (212–300 μm) in a Fastprep homogenizer after addition of 8% (vol/vol) $HClO_4$, neutralization with 1.3M $KHCO_3$, and buffering with 1M K phosphate (pH 7.6) (B. P. Braeckman, personal communication, adapted from Braeckman *et al*. [2002]). The ATP bioluminescence CLSII kit (Roche, Manheim, Germany) was used to measure the *in vitro* ATP levels and the BCA Protein Assay kit used (Pierce, ThermoFisher Scientific Inc., Rockford, IL) to determine the protein levels in extracts (as mg/ml). ATP concentrations were normalized to protein content of samples.

Data analysis. Results were expressed as a percentage of the values obtained for control conditions (0μM Cd). Error bars represent SEM. Dose-response curves were analyzed by nonlinear regression analysis applied to individual data points using SigmaPlot Version 11. Bioluminescence, *in vitro* luciferase activity, and reproduction data were fitted to sigmoidal, Gompertz model curves (Vulkan *et al.*, 2000). The equation used was $y = y_0 + a\exp[-\exp\{-b(x - x_0)\}]$, where y is the percent response (bioluminescence, *in vitro* luciferase activity) or the ratio of L1 larvae to adults (reproductive output), x is the Cd concentration, and y_0, x_0, a, and b are model parameters fitted to data. The *in vitro* ATP normalized to protein decayed according to an exponential function with equation $y = y_0 + a\exp(-bx)$, where y_0, a, and b are model parameters. The protein levels followed a sigmoidal logistic four-parameter model. Adjusted r^2 values are shown for significant relationships (ANOVA, $p < 0.001$). The Cd concentrations that decreased the response by 20% (EC_{20}) have been estimated from the fitted functions and are shown when appropriate. Mean values and the SEM only are shown for fluorescence data as they were not well described by mathematical functions.

RESULTS

Bioluminescence and Feeding Response of Strain PE322

Cd exposure experiments (19 h) focused on 0–30μM Cd as the sublethal range.

These exposure conditions produced negligible lethality. Exposure to 100μM Cd gave rise to less than 5% lethality, but nematodes were immobile and smaller. Exposure to 200 and 400μM Cd gave rise to approximately 8% and 13% lethality, respectively (data not shown).

The differences in the condition of nematodes observed by microscopy after 19-h exposure to 0–30μM Cd were subtle: a decrease in activity as judged by movement and a "thinner" and smaller appearance with increasing Cd concentrations. Nevertheless a quantitative concentration-dependent decrease in bioluminescence of nematodes was observed upon exposure to 0–30μM Cd (Fig. 1A). A decrease in bioluminescence was observed at Cd concentrations as low as 10–15μM, for which no clear visual differences in the condition of nematodes was apparent. The concentration of Cd estimated to lead to a 20% reduction in bioluminescence (EC_{20}) was 8.2 and 13.8μM in repeated experiments. These experiments were carried out with strain PE322, which carries the *luc::gfp* genes extrachromosomally, and therefore, approximately only 20% of the nematodes were *luc::gfp* marked (and displayed a more heterogeneous pattern of transgene expression). In subsequent experiments, the chromosomally integrated strains (PE254 and PE255) were used. Reduced bacterial consumption by nematodes exposed to Cd concentrations above 10 or 15μM was indicated by the increased optical density at 550 nm (OD_{550}) of the bacterial suspensions (Fig. 1B).

Bioluminescence, Fluorescence, and Luciferase Activity Levels of Strains PE254 and PE255

In addition to the bioluminescence response (*in vivo*), we measured fluorescence as an indication of the expression levels of the green fluorescent protein (GFP)-tagged luciferase and the *in vitro* luciferase activity levels of Cd-exposed nematodes, to determine how these parameters were affected by exposure to Cd (Fig. 2). Following exposure to 30μM Cd, all three parameters were reduced to approximately 44–58% (bioluminescence), 56–58% (GFP fluorescence), and 68–69% (*in vitro* luciferase activity, not normalized to protein) of control levels. Further reduction in bioluminescence was only observed for Cd concentrations (> 30μM Cd) which incurred significant lethality (data not shown). Fluorescence and *in vitro* luciferase activity declined more gradually than bioluminescence up to approximately 15μM Cd exposure. The concentration of Cd estimated to lead to a 20% reduction in *in vitro* luciferase activity (EC_{20}) was similar in experiments with PE254 or PE255, 23.7 and 22.6μM Cd, respectively. In

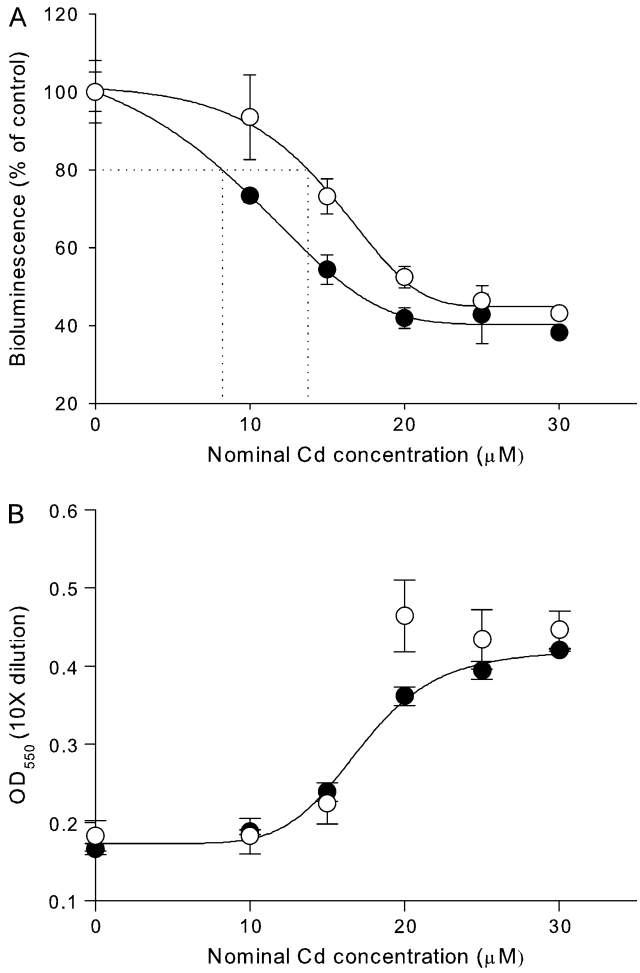

FIG. 1. Response of *luc::gfp*-marked *Caenorhabditis elegans* (strain PE322) to sublethal Cd concentrations (19-h exposure). (A) Mean bioluminescence, expressed as a percentage of 0μM Cd data, decreased with increasing Cd concentrations. (○,●) Duplicate independent experiments carried out at different times. Dose-response curves are described by nonlinear regression (see data analysis; ○, $r^2 = 0.8922$; ●, $r^2 = 0.9049$; ○, ●, $p < 0.001$). Drop lines show the estimated Cd concentrations that cause a 20% reduction in bioluminescence (EC_{20}). (B) The OD_{550} of test solutions (10× diluted) increased with increasing Cd levels. ●, $r^2 = 0.9799$; $p < 0.001$. Error bars depict SEM ($n = 3$).

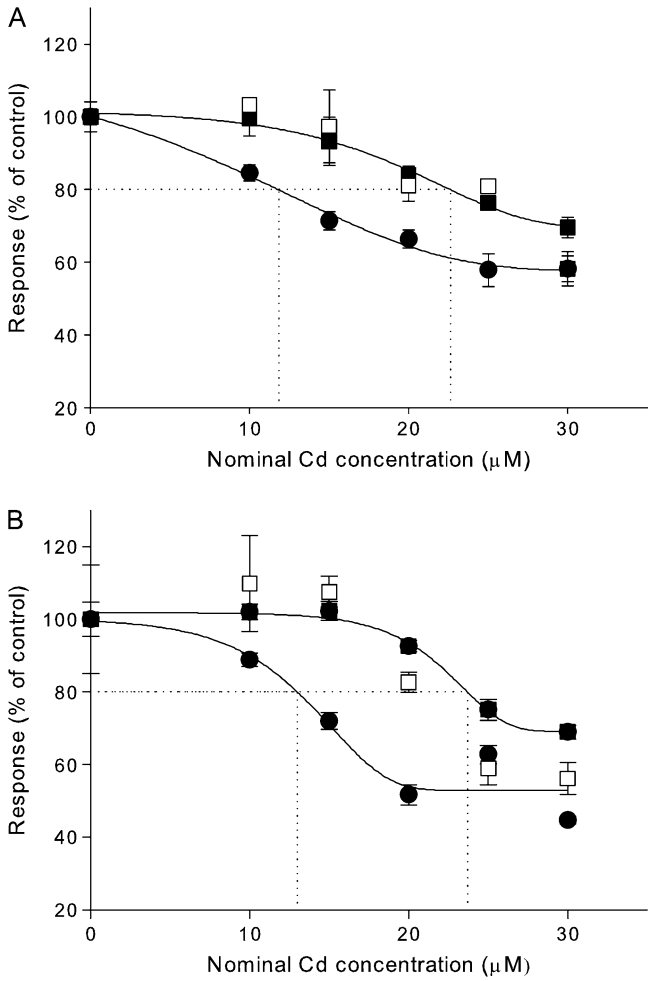

FIG. 2. Response to sublethal Cd concentrations (19-h exposure) as measured by bioluminescence (●), GFP fluorescence (□), and *in vitro* luciferase activity (■) of (A) strain PE255 and (B) PE254. Results expressed as a percentage of controls exposed to 0μM Cd. The relationship between each response and the Cd concentration was described by nonlinear regression (see data analysis) ([A] ●, $r^2 = 0.9050$; ■, $r^2 = 0.8168$; ●, ■, $p < 0.001$; [B] ●, $r^2 = 0.8718$; ■, $r^2 = 0.9204$; ●, ■, $p < 0.001$). Drop lines show EC_{20} values. Concentrations of Cd ≤15μM lead to a more pronounced decline in bioluminescence than in GFP fluorescence or luciferase activity. Mean values and the SEM only are shown for fluorescence as data were not well described by mathematical functions (as indicated by examination of residuals). Experiments with the strains PE255 and PE254 were carried out independently and at different times. Error bars indicate SEM ($n = 3$).

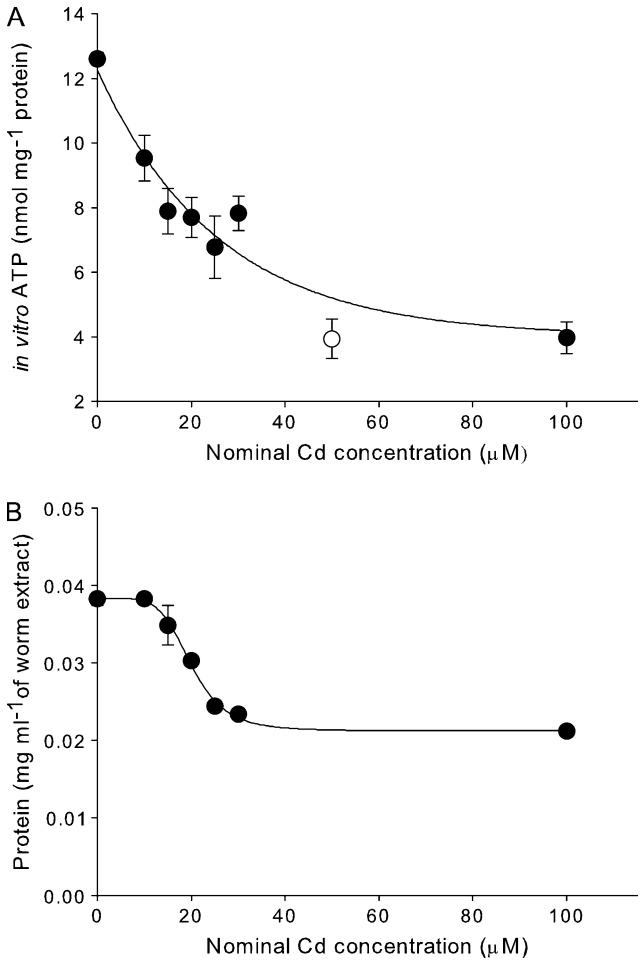

FIG. 3. Effect of Cd exposure (19 h) on (A) the *in vitro* ATP levels normalized for protein content and (B) relative protein levels of N2 *C. elegans*. Normalized ATP levels of Cd-exposed nematodes declined exponentially in a concentration-dependent manner, as described by nonlinear regression (see data analysis) ($r^2 = 0.7547$; $p < 0.001$). In (A), data for 50μM Cd (○) were not included in trendline because estimated protein values (from trendline in [B]) were used to normalize ATP measurements. The decline in protein levels with increasing Cd followed a logistic function ($r^2 = 0.9485$; $p < 0.001$). Representative experiments are shown. These experiments were reproduced independently twice and the same concentration-response trends obtained. Error bars indicate SEM ($n = 4$).

contrast, a lower EC_{20} for bioluminescence was estimated (13.0 and 11.8μM Cd, respectively, for PE254 and PE255) which reflected the significant decrease in bioluminescence at low Cd concentrations (<15μM). Luciferase activity normalized to protein content did not decrease with increasing Cd concentrations up to 30μM (Supplementary data), indicating that Cd exposure did not affect enzyme activity directly.

The amount of luciferase present, or its activity levels, was not sufficient to explain the drop in bioluminescence observed, particularly following exposure to concentrations ≤15μM Cd. We postulate that perturbation of ATP levels of Cd-exposed nematodes accounts for the sensitive bioluminescence response to these sublethal Cd levels.

In Vitro ATP and Protein Levels

To verify the relationship between perturbation of ATP levels and changes in the bioluminescence response (*in vivo*), we carried out *in vitro* determination of ATP levels in Cd-exposed N2 wild-type worms. The N2 strain was selected rather than a transgenic bioluminescent strain so that luciferase was not present in the nematodes' tissues which may have interfered with the performance of the *in vitro* ATP kit used. Exposure to Cd resulted in significant concentration-dependent decrease in ATP per milligram of protein (Fig. 3A). The

protein levels (mg/ml of extract) *per se* also showed significant concentration-dependent decline (Fig. 3B). A very pronounced drop in protein levels was observed between 15 and 25μM exposure, comparatively little further decrease occurred up to 100μM Cd. Exposure to 0–15μM Cd did not result in a significant change in protein levels, in agreement with GFP fluorescence and *in vitro* luciferase activity measurements (Fig. 2) which remained largely the same following exposure to 0–15μM Cd. However, a marked decrease in *in vitro* ATP levels per milligram of protein occurred in the 0–15μM Cd range (Fig. 3A) consistent with the observed decrease in bioluminescence (Fig. 2).

Development and Reproduction

In order to assess the usefulness of bioluminescence as a means of detecting sublethal effects of Cd exposure, we determined how nematode development and reproductive output fared as experimental end points of sublethal Cd exposure.

A concentration-dependent slowdown in development was observed after 72-h exposure to sublethal Cd levels initiated at the L1 stage (Fig. 4). Most of the nematodes in controls (0μM Cd) reached adulthood, approximately 6% developed only into the L4 stage. Marked differences in the stage of development attained were observed at 20μM Cd and above. For 20μM Cd, a decrease in the proportion of adults present after 72-h exposure was accompanied by an increase in the nematodes at the L4 stage, with a very small proportion of worms at the L3 stage. For 30μM Cd, only mixed populations of L4 and L3 larval stages were present. Whereas, exposure to 100μM Cd had a severe impact on development and no worms developed further than the L1-L2 larval stage. Developmental delay proved an informative end point in toxicology research; however, these assays are longer than bioluminescence assays (72 vs. 19 h), more time consuming and laborious (visual observation vs. automated measurements).

Reproductive output was assessed as the number of L1 progeny per adult worm. The total number of worms per test well at the end of experiments was under 100,000 and an OD_{550} of approximately 0.2 was present in the 0μM Cd controls (data not shown). Exposure (67 h) of 36-h posthatch (late L3 larval stage) to 10–30μM Cd led to significant concentration-dependent reduction in the number of progeny (Fig. 5). Reproductive output proved a very sensitive end point with an EC_{20} of 10.2μM Cd. Very few progeny were observed for concentrations of 20μM Cd and above. Reproduction and bioluminescence end points showed comparable sensitivity (EC_{20} of 10.2μM and 11.8–13μM Cd, respectively); however, exposure time was 67 h in reproduction experiments as compared to the 19-h exposure of bioluminescence assays.

DISCUSSION

In this study, we tested *C. elegans* bioluminescence as a direct measure of metabolic status in chronic sublethal toxicity assays, using Cd as a model toxin. The concentration-dependent response to Cd could be divided into two stages, ≤15μM Cd and 15–30μM Cd. Up to approximately 15μM Cd, the metal had little or no effect on luciferase levels and total protein values but reduced bioluminescence in line with a reduction in *in vitro* ATP levels (Fig. 3A). This ATP decrease produced by Cd exposure is likely to have resulted from impaired mitochondrial function, combined with the high energetic cost of mounting a stress response. Cd exposure is

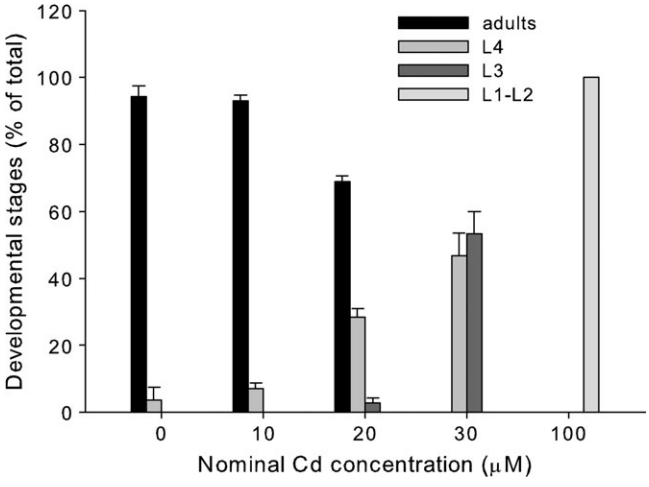

FIG. 4. Developmental stage attained following 72-h exposure to the cadmium levels indicated. A synchronized population of L1 stage worms (strain PE322) was present at onset of experiments. Cd elicits a concentration-dependent slowdown in development. Three independent batches of worms were used in experiment. Error bars indicate SEM ($n = 3$).

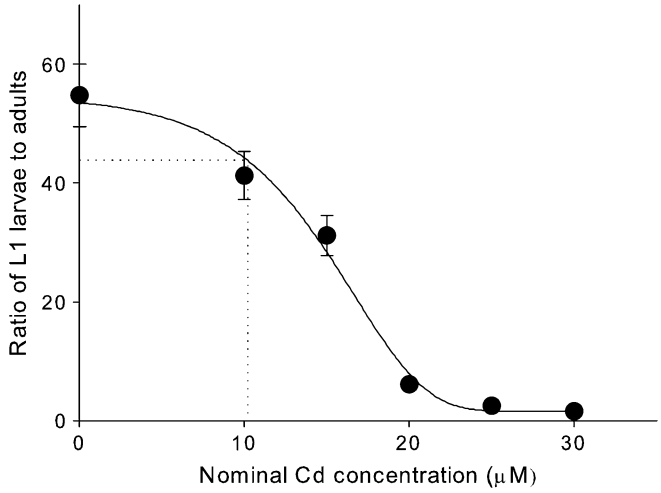

FIG. 5. Average number of L1 larvae produced per adult following 67-h exposure to Cd initiated at the late L3 larval stage (36-h posthatch). Sublethal levels of Cd led to a significant decline in reproductive output, described here by nonlinear regression (see data analysis; $r^2 = 0.8764$; $p < 0.001$). Drop line indicates EC_{20}. Three independent batches of worms were used in experiment. Error bars indicate SEM ($n = 3$).

known to lead to mitochondrial dysfunction by multiple mechanisms which include uncoupling of oxidative phosphorylation (Jacobs *et al.*, 1956), reduction of electron transport chain activity (Miccadei and Floridi, 1993), inhibition of various enzymes of oxidative metabolism (Ivanina *et al.*, 2008), indirect generation of reactive oxygen species via depletion of antioxidants (Bertin and Averbeck, 2006), and induction of the mitochondrial permeability transition (Rikans and Yamano, 2000).

An increase in energy demand associated with the induction of the stress response is also likely to have contributed to the ATP drop observed, which was detectable by both luminescence (Fig. 2) and *in vitro* ATP determination (Fig. 3) for the lowest Cd concentration tested of 10μM. Biosensors that report on stress-inducible gene transcription in transgenic nematodes indicated that stress-responsive mechanisms are activated in the range of Cd exposure conditions that we tested: induction of the heat shock response occurs upon 5-h exposure to Cd at concentrations as low as 8.9μM (Candido and Jones, 1996) and induction of metallothioneins upon 24-h exposure to 0.1–2.5μM Cd (Cioci *et al.*, 2000; Swain *et al.*, 2004). Interestingly, in spite of the increased energetic costs of exposure to 10μM Cd, this was not sufficient to markedly affect growth and development as assessed by protein levels (Fig. 3B) and in developmental experiments (Fig. 4). The lowest concentration of 10μM Cd had a clear effect on reproduction, indicating that this end point is of comparable sensitivity to bioluminescence. A level of Cd of 10μM has been shown to cause increased levels of germ cell apoptosis in *C. elegans* (Wang *et al.*, 2008), which may explain the lower fecundity.

Above 15μM Cd, in addition to a reduction on ATP levels, experiments revealed a reduction in the levels of the enzyme luciferase concomitant with a decrease in the total protein content and a small but detectable reduction in the size of worms (not quantified in this study). These observations are in agreement with several studies that showed significant growth reduction in Cd-exposed nematodes (Höss *et al.*, 2001; Popham and Webster, 1979; Traunspurger *et al.*, 1997; van Kessel *et al.*, 1989). Our experiments indicated that the bioluminescence response to 15–30μM Cd exposure (19 h) resulted from a combined effect of Cd on ATP levels and on luciferase protein levels (ensuing from reduced growth). A strong effect of Cd on development (and consequently growth) above 15μM Cd was independently shown by the developmental experiments we carried out (Fig. 4).

Cd interferes with both food uptake (Boyd *et al.*, 2003; Jones and Candido, 1999; Swain *et al.*, 2004) and the assimilation of energy from food and has been proposed to render the nematodes in a state of "physiological starvation" (Popham and Webster, 1979). Jones and Candido (1999) determined that 17.8μM Cd (2 ppm) was the lowest Cd concentration to cause detectable decrease in the nematode's rate of consumption of *E. coli* over a 5-h period. In this study, we demonstrated an effect of sublethal Cd on ATP levels (both by bioluminescence and *in vitro* ATP determination) and at sublethal concentrations above 15μM, an effect on nutrient intake via a reduction in feeding (as assessed by bacterial density in test suspensions) and on growth-related parameters (protein levels, luciferase levels). We have also shown an overall correspondence between the levels of Cd that impacted bioluminescence (following 19-h exposure) and those that had an impact on development and reproductive output after 72 and 67 h tests, respectively. Therefore, we conclude that bioluminescence of *C. elegans* is a good predictor of Cd effects on development and reproduction. Our results are in agreement with previous work reporting that growth and development are dependent on mitochondrial respiratory chain activity (Tsang and Lemire, 2002).

It is likely that the link between Cd effects on energy and nutritional status and its subsequent impact on growth, development, and reproduction are mediated by target of rapamycin (TOR), adenosine-5′-monophosphate (AMP)-activated protein kinase (AMPK), and insulin signaling. TOR kinase signaling regulates mRNA translation, ribosomal biogenesis, and protein synthesis, processes on which growth and development depend. TOR deficiency results in slower development, reduced growth, and marked gonadal degeneration in *C. elegans* (Jia *et al.*, 2004; Long *et al.*, 2002), reduced body size in *Drosophila* (Zhang *et al.*, 2000), and slower growth and lower seed production in *Arabidopsis* (Deprost *et al.*, 2007). Physiological conditions that decrease TOR activity include low levels of amino acids, reduced insulin signaling and/or the activation of the AMPK by cellular stress conditions that increase the AMP/ATP, ensuing inhibitory effects on translation and growth (Avruch *et al.*, 2006). We envisage that our bioluminescent assay in conjunction with the genetic tools developed for *C. elegans* will contribute to a better understanding of these fundamental metabolic sensing mechanisms and their role in the response to toxins.

Future work will apply our bioluminescent sublethal assays to characterize the biological effects of stress and protective mechanisms employed to combat these, particularly when toxins are present in complex mixtures such as in soils, sediments, or sewage sludge. The sensitivity of bioluminescence as an end point is demonstrated by the maximum concentration of 30μM Cd used in this study being approximately 250× lower than the reported 24-h Cd LC_{50} (the concentration causing 50% lethality) for *C. elegans* (Williams and Dusenbery, 1990) and 4–6× lower than the 24-h EC_{50} for sublethal parameters such as movement, feeding, growth, and reproduction (Anderson *et al.*, 2001). A strong advantage of bioluminescence over other toxicological end points is that assays for metabolic status can be conveniently and rapidly carried out in the laboratory in a 96-well plate format, enabling high-throughput toxicity screening. In contrast, the detection of adverse effects of Cd on sublethal parameters such as growth, development, and reproduction, typically required long

exposure times (72–3.5 days) (Hoss *et al*., 2001; Popham and Webster, 1979; Traunspurger *et al*., 1997; van Kessel *et al*., 1989).

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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