The effect of biological and chemical factors on the uptake and toxicity of cadmium in the duckweed Lemna trisulca L.

by

David B. Huebert

A thesis presented to the University of Manitoba
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Botany

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DAVID B. HUEBERT

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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ABSTRACT

The duckweed Lemna trisulca L. was grown in axenic cultures in a filter-sterilized medium. A portion of the medium was replaced regularly during experiments. The amount replaced doubled every two days. Metal concentrations in the medium were measured on the last day of experiments. The only organic compound added was FeEDTA at a concentration of 9 µM. The chelating capacity of the medium was therefore minimal, defined and controlled. The nutrient medium and environmental conditions supported a doubling time of 1.6 to 2.4 days over a 14 day culture period.

Under the above conditions, the EC50 (concentration at which a 50% effect is observed) for Cd was 0.99 µM based on multiplication rate and 0.56 µM based on final yield. The NOEC (no observable effect concentration) for Cd was between 120 and 150 µg Cd/g dry wt. based on internal Cd, and 0.08 µM based on external Cd. Internal Cd may be a superior estimate of toxicity because it avoids the problems associated with metal speciation in the external medium and allows for a comparison with field data.

Lemna trisulca responded within 2 days to the addition of 0.64 µM Cd. No tolerance to Cd was induced even after 6 weeks of exposure. The calculated response of L. trisulca to Cd may have been influenced by the multiplication rate of control cultures, which varied from 1.6 to 2.4 days.

Calcium had no effect on Cd uptake or toxicity. Zinc antagonized Cd toxicity but had a variable effect on Cd uptake depending on its concentration. Zinc was about fifteen times less toxic than Cd. The uptake and toxicity of Cd and Zn were almost completely prevented when the level of available EDTA was in excess of the Cd or Zn concentration.

These data indicate that biological and chemical factors can profoundly influence the effect of toxicants on living organisms. These factors must be considered to ensure that toxicity studies are not confounded by effects extraneous to the actual effect of the toxicant.

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CHAPTER 1. Introduction

Rationale for the study

Natural biogeochemical cycles of trace metals have been swamped by anthropogenic inputs. For example, the global annual input of Cd into the biosphere from natural sources such as volcanoes, forest fires and soil weathering has been estimated at about 4,500 tonnes/year (Nriagu and Pacyna, 1988; Nriagu, 1990). In contrast, total industrial discharges of Cd have been estimated at 24,000 tonnes/year (Nriagu and Pacyna, 1988; Nriagu, 1990). Of this, an estimated 9,100 tonnes/year is released into aquatic ecosystems. These alarming statistics make it imperative that we understand the effects of trace metals such as Cd on natural ecosystems so we can protect these ecosystems in the future. Freshwaters are particularly sensitive to trace metal inputs because many are of relatively small size, and have low ambient metal concentrations and relatively high mobility of trace metals (Nriagu, 1990).

This thesis examines the effects of chemical and biological factors on Cd uptake and toxicity in the freshwater plant Lemna trisulca L. A primary reason for the choice of study is that Cd is of concern because of its persistence, mobility and toxicity in the aquatic environment. Once Cd is dispersed into aquatic ecosystems it cannot be degraded or recovered (Nriagu, 1990). The aquatic vascular plant L. trisulca was chosen because of the relative lack of information on the uptake and toxicity of metals in these plants. This information is important because aquatic plants form the basis of food chains and provide habitats for other species. Aquatic plants, particularly Lemna spp., are being used increasingly in bioassay protocols designed to determine water quality guidelines (U. S. EPA, 1985; Hughes et al., 1988; Taraldsen and Norbert-King, 1990; Wang, 1990; ASTM, 1991; Wang, 1991). If these protocols are to be effective, accurate and precise

laboratory data on the toxicity of metals such as Cd to Lemna are of paramount importance.

Unfortunately, available information on the toxic effects of Cd on aquatic plants is inconsistent (Outridge and Noller, 1991). For instance, Cd EC50 values (the concentration causing a 50% reduction in a tested variable) range from 0.09 to about 1,350 µM (Outridge and Noller, 1991). An understanding of the reasons for this inconsistency is critical if accurate and precise information on metal uptake and toxicity in aquatic plants is to be obtained.

Description of Lemna trisulca L.

Lemna trisulca is an aquatic vascular plant of the Class Monocotyledonae, Order Arales, Family Lemnaceae. It is thought to be closely allied to the genus Pistia in the family Araceae (Arber, 1920; Sculthorpe, 1967). Lemna trisulca, commonly called star duckweed or ivy-leaved duckweed, is a submerged aquatic macrophyte that has roots but grows unattached to the substratum. On the basis of this growth form, Hutchinson (1975), classifies it as a mesopleustophyte or megaloplankton.

The extremely small size of L. trisulca and other species of the duckweed family has resulted in difficulties in interpretion of the various vegetative parts of the plant. Lemna trisulca does not possess distinct leaves and stems. The most widely held view is that the frond of L. trisulca is a combined stem and leaf; the proximal end is axial and the distal end is foliar (Sculthorpe, 1967; Landolt, 1986). Fronds are 4 to 15 mm long and 2 to 7 mm wide (Gleason, 1952; Clapham et al., 1957). They are translucent, oblong to narrowly ovate, acute at the apex and three-veined. Vascular bundles are reduced and not lignified. They contain one xylem lacuna, one sieve tube and two companion cells (Sculthorpe, 1967). Fronds are attached to each other by persistent, long-stalked (4 to 16 mm) connections (Gleason, 1952) which produce extensive tangled mats. Roots, when

present, occur one per frond, and are variable in length, reaching up to 50 mm (pers. obs.). The roots of L. trisulca have conspicuous root caps but no vascular tissue. Fertile fronds of L. trisulca are smaller than vegetative ones, are pale green in color, float at the water surface and contain stomates. Flowers are minute, unisexual and lack a perianth (Sculthorpe, 1967). They arise from lateral pouches in the fertile frond. The fruit is a utricle and the seed is approximately 1 mm in diameter. The diploid chromosome number ranges from 20 to 80 (Landolt, 1986). Turions are not formed (Landolt, 1986).

Lemna trisulca is a clonal organism. The modular unit consists of a frond of determinate size with a single root. The plant grows by producing two new fronds which arise from two small pouches on opposite sides of the parent frond, and at right angles in the same plane as the parent. Unlike most other clonal plants which spread in two dimensions across a substratum, however, L. trisulca spreads in three dimensions within the water column. This is accomplished by a 90° twist in the developing stem which results in the orientation of any three contiguous fronds in all three dimensions (pers. obs.). The growth of the plant is rapid, with a reported doubling time of 3 days (Landolt, 1957).

The distribution of L. trisulca is subarctic circumpolar. It grows in cooler climatic zones around the world except in South America and most Pacific Islands, and has been found as far as 69° N (Landolt, 1986). Lemna trisulca is found in still waters of ponds, ditches, stream margins, swamps and lakes (Scoggan, 1957, 1978). It is a bicarbonate user (Hutchinson, 1975; Spence and Maberly, 1985) with a relatively high demand for Ca, Mg, K and C (Landolt, 1986) and is most often found in moderately hard, carbonate-rich waters (Hutchinson, 1975; Landolt, 1986). It is absent from oligotrophic or excessively eutrophic waters (Landolt, 1986). Duckweeds, including L. trisulca, provide food, habitat or cover for beaver, muskrat, racoons, ducks, geese, turtles, fish, crustaceans, gastropods and insects (Landolt, 1986). Lemna trisulca occurs in habitats where it must compete with duckweeds and other floating macrophytes and suspended

algae (Landolt, 1986; McIlraith et al., 1989). It is found in conjunction with Ceratophyllum demersum L., Myriophyllum spicatum L. and emergents such as Typha spp, Phragmites australis (Cav.) Trin ex Steudel, Scirpus spp. and Carex spp. (Landolt, 1986).

Lemna trisulca is an excellent organism for physiological, biochemical and toxicological studies. Considerable amounts of plant material can be obtained quickly and in a limited space because of its rapid growth and small size. Uniform vegetative material can be maintained for extended periods since it is a clonal organism. It is non-rooted, which means that fronds can be relatively easily sterilized and grown in axenic cultures in aqueous media. Unlike other species of duckweeds, however, L. trisulca is a submerged aquatic plant which, because its vegetative fronds lack stomates and a cuticle, obtains its inorganic carbon from the water. Its free-floating, submerged habit is an advantage in aquatic toxicity studies because it avoids problems associated with air/water and sediment/water interfaces. These characteristics were the main reason why L. trisulca was chosen for this study.

Cadmium

Cadmium is a silvery white, soft, ductile metal with a bluish tinge. Cadmium is a IIB metal with an atomic weight of 112.4 and an atomic number of 48. Its neighbours in the periodic table are Zn and Hg. The concentration of Cd in the lithosphere ranges from 0.1 to 0.2 µg/g, which makes it a rare element (Nriagu, 1980). It forms sulfide minerals and typically is found as impurities in other sulfide minerals such as ZnS_2. The average Cd content of ZnS_2 is 0.02 to 1.4% with a median of about 0.3%. It is never mined in isolation, but is purified as a byproduct when Zn, Pb, Zn-Pb, Zn-Cu or complex ores are smelted.

Cadmium is used primarily in electroplating, pigments, batteries and as a plastics stabilizer (Nriagu, 1980). Of the total amount of Cd consumed in industry, electroplating accounts for about 35%, pigments for about 25%, and batteries and plastics for about 15% each. Nriagu (1980) refers to these uses as 'dissipative', since less than 5% of Cd is recycled after being used in these four processes.

As mentioned above, the worldwide emission of Cd into aquatic environments from anthropogenic sources has been estimated at 9,100 tonnes/year (Nriagu, 1990). Manufacturing processes account for 2,400 tonnes/year, atmospheric fallout (mostly from smelting and refining of metals) accounts for 2,200 tonnes/year, base metal mining and smelting account for 2,000 tonnes/year and domestic waste adds about 1,700 tonnes/year (Nriagu and Pacyna, 1988; Nriagu, 1990). The inputs into aquatic enviroments are about twice the amount of Cd mobilized naturally in the biosphere from such sources as volcanoes, forest fires and soil weathering (Nriagu, 1990).

The chemical properties of Cd are such that in oxygenated waters with a pH less than 8.0, Cd will exist mostly as the free divalent cation (Khalid, 1980). Above pH 8.0, Cd carbonate and hydroxy complexes become increasingly dominant. In anoxic conditions, CdS_2 is the major component formed (Khalid, 1980). The complexation of Cd to organic compounds is more variable. Giesy (1980) reported that the amount of Cd complexed by dissolved organic carbon ranged from 17 to 90% in freshwater and from 0 to 80% in seawater. This wide range is caused by the heterogenous nature of humic and fulvic substances, by the complex mixture of organic ligands and by the difficulty in determining molar ligand concentrations in natural waters. These conditions make it difficult to calculate overall formation constants in natural waters (Giesy, 1980) and therefore difficult to determine accurate metal speciation in freshwaters. An accurate knowledge of metal speciation is important, because it is likely that the toxic effect of Cd and other metals is due to their free ion activity and not to their total concentration,

(Anderson and Morel, 1978; Borgmann, 1983; Hall, 1986; Nor and Cheng, 1986; Borgmann et al., 1991).

Although Cd was purified and named in Germany in 1817, it was known to be toxic long before that time (Nriagu, 1980). Cadmium has produced toxic effects in some aquatic organisms at concentrations as low as 0.2 µg/L (Borgmann et al., 1991; Lawrence and Holoka, 1991). Nriagu (1990) states that about 250,000 to 500,000 people worldwide suffer from renal dysfunction caused by Cd poisoning. In Japan, about 9.5% of rice paddies have been taken out of production because of excessive metal levels, which presumably also include Cd. Cadmium is also bioaccumulated. High Cd content is one of the reasons why the kidney and liver of moose, deer and bear in central Canada are unsuitable to be eaten (Nriagu, 1990). Cadmium is persistent in the environment since it cannot be degraded or recovered once it has been released. If current anthropogenic mobilization rates continue, the toxicity, bioaccumulation and persistence of Cd in the environment make it potentially one of the most serious contaminants.

The effect of biological and chemical factors on metal uptake and toxicity

The following section will discuss the interaction between metal uptake and toxicity, and biological and chemical factors. It is important to understand these interactions because they may significantly alter metal effects and lead to errors of interpretation of toxicity data. The discussion will begin with the effect of biological factors such as the growth medium, induction of metal tolerance and how toxicity may change over time. This will be followed by a discussion on the effect of chemical factors on metal toxicity; most notably how EDTA alters uptake and toxicity.

Few studies have examined the effects of biological factors such as the growth medium on metal uptake and toxicity in aquatic plants. Plants that are growing poorly in an unsuitable medium may react to a toxicant in a different manner from plants growing

vigorously under optimal conditions. Landolt and Kandeler (1987), for instance, state that no nutrient media exist that are specifically designed to grow duckweeds. Lemna trisulca, as an example, is purported to require glucose, vitamins, low light and an acidic pH for successful growth in the laboratory (Landolt, 1957; Bowker et al., 1980). Under natural conditions, however, it grows at an alkaline pH in relatively high light.

Little agreement exists regarding the optimum length of time for toxicity tests using duckweeds. Landolt and Kandeler (1987) state that duckweeds should be grown for four weeks under constant conditions before any growth rate measurements are taken in order to accommodate the lag between uptake and toxicity. Toxicity tests that use duckweeds, however, are only of 4 to 7 days duration (U. S. EPA, 1985; Hughes et al., 1988; Taraldsen and Norberg-King, 1990; Wang, 1990; ASTM, 1991; Wang, 1991). Using Lemna minor L., Kwan and Smith (1991) showed that the Cd content reached steady state conditions after approximately 12 days when the plant was exposed to 1.5 μM Cd. This suggests that perhaps four weeks is too long but that 4 to 7 days is not long enough to adequately assess toxicity. Toxicity will be underestimated if the lag period constitutes a large portion of the test period.

Metal tolerance may also be induced in aquatic plants. Outridge and Hutchinson (1991) and Outridge et al. (1991) have studied the induction of Cd tolerance in Salvinia minima Baker and have found that the concentration of phytochelatins increased significantly upon exposure to Cd. Phytochelatins are proteins that can bind toxic metals (Rauser, 1990) and thus may be important in metal detoxification. If metal tolerance is induced, then the degree of toxicity may depend on the length of exposure to the toxicant.

Variations in the growth rate of control cultures over time and interactions between the control growth rate and the effect of a toxicant must also be considered. Studies have found that the intrinsic growth rates of aquatic plants are not constant over time (Huebert and Gorham, 1983; Landolt and Kandeler, 1987; Thorsteinsson et al., 1987; Wang, 1990). Wang (1990) found that the doubling time of L. minor varied from 1.3 to 2.8 days

over an 18 month period. Data on variations in the effect of a toxicant over time are rarer. Wang (1990) reported no changes in the effect of Cr on L. minor over time, but unfortunately did not examine the relationship between intrinsic growth rate and the Cr reference toxicant. Seasonal fluctuations in growth rate or relationships between growth rate and the effect of a toxicant could confound efforts to assess toxicity. In summary, the effects of biological factors on metal toxicity is an area that needs further study if any confidence is to be placed in metal toxicity data.

Effects of chemical factors on metal uptake and toxicity are considerably better understood (Landolt and Kandeler, 1987; Outridge and Noller, 1991). Metals in aquatic environments occur in a complex matrix of other mineral nutrients and organic compounds. It is necessary to understand interactions between metals and these chemical compounds in order to be able to predict metal toxicity in the environment and to design valid laboratory test protocols.

One of the most important interactions is between metals and dissolved organic carbon compounds such as chelators. This is because chelators reduce free ion activity and, as already mentioned, it is probably the free ion activity and not the total metal concentration which determines metal toxicity (Anderson and Morel, 1978; Borgmann, 1983; Hall, 1986; Nor and Cheng, 1986; Borgmann et al., 1991). Over the last thirty years, there has been considerable work on the effect of chelators on metal uptake and toxicity. One of the first studies was by Hillman (1962) who found that the chelator ethylenediaminetetraacetic acid (EDTA) had a profound effect on Cu toxicity. He observed that 30 μM EDTA added to the culture medium reversed the effect that excess Cu had on flowering in Lemna perpusilla Torrey and Lemna gibba L. In an attempt to explain this reversal, Tanaka et al. (1982) and Nasu et al. (1983) repeated the experiment twenty years later. They found that adding 30 μM EDTA to the medium prevented the uptake of Cu. In a medium with no added EDTA and 20 μM Cu, the internal Cu content in Lemna paucicostata (Hegelm.) reached approximately 2,000 μg Cu/g dry wt. This was

reduced to negligible levels upon the addition of 30 µM EDTA. Similarly, the internal Cu content of L. gibba was approximately 500 µg Cu/g dry wt. at 4 µM Cu in the absence of EDTA. This was reduced more than 90% when 30 µM EDTA was included in the medium. The addition of 30 µM EDTA also completely antagonized the effect of up to 20 µM Cu on final yield (mg dry wt.). The results of Hillman (1962) were thus explained on the basis of an antagonistic effect by EDTA on Cu absorption.

The chelator EDTA was also found to antagonize Cu uptake and toxicity in Spirodela polyrhiza (L.) Schleiden (Schreinemakers and Dorhout, 1985). The concentration of EDTA in their study ranged from 6.7 to 400 µM. The EC50 for Cu increased from 4.5 µM Cu at 6.7 µM EDTA to 310 µM Cu at 400 µM EDTA. There was a linear relationship between the external EDTA concentration and the EC50 for Cu. Their most intriguing finding was that the slope of the line was less than 1.0 (with EDTA plotted on the abscissa). A slope of 1.0 is expected because there is a 1:1 stoichiometry between EDTA and chelated Cu (Skoog and West, 1969). The lower slope means that as the EDTA concentration increased, the EDTA was less efficient at chelating Cu. The reason for this remains obscure.

Nor and Cheng (1986) used Eichornia crassipes (Mart.) Solms in a study of the effect of various ligands, including EDTA, on Cu uptake. They concluded that Cu bound to EDTA was unavailable for uptake. Consequently, this suggested that it was the level of free Cu^{+2} ions that governed uptake. In their study they used 30 µM Cu, and found that an EDTA:Cu ratio of 3.0 was needed before Cu absorption was prevented. Under the conditions of their study, greater than 95% of total Cu was absorbed by the plant in the absence of EDTA. In contrast, simple chelators such as glycine, alanine, citrate and tartrate were not nearly as effective as EDTA in preventing Cu absorption. Nor and Cheng (1986) also examined the effect of fulvic and humic acids on Cu absorption. The fulvic acid (FA) fraction, extracted from a soil of unknown origin, had no effect on Cu absorption. The humic acid (HA) fraction, however, completely prevented Cu absorption,

even in the presence of 150 μM Cu. These data suggest that, depending at least in part on the FA/HA ratio, different soils and water samples have variable chelating properties at the same DOC level. This is consistent with the work of Garvey et al. (1991), who showed that different soil extracts had variable chelating properties.

In contrast to those with Cu, results on the effect of EDTA on Cd uptake and toxicity are inconsistent. In one study (Nasu et al., 1983), Cd uptake and toxicity were unaffected by the addition of 30 μM EDTA. Regardless of the presence or absence of EDTA, when 20 μM Cd were present in the medium, the duckweeds L. gibba and L. paucicostata contained 2,000 to 3,000 μg Cd/g dry wt. and the final yield was reduced by approximately 80%. In a second experiment, however, Cd uptake was reduced by approximately 25% at 30 μM EDTA, 60% at 100 μM EDTA and 90% at 200 μM EDTA. Interestingly, there was no concomitant increase in the final yield as the Cd content within the plants fell in response to increased EDTA. These results are difficult to interpret.

Fayed and Abd-el-Shafy (1985) examined the effect of EDTA on Cd uptake in E. crassipes . In one experiment they grew plants in the presence or absence of 8.9 μM Cd and 400 μM EDTA. Cadmium uptake was unaffected by the presence of 400 μM EDTA. Plants contained approximately 900 ± 400 μg Cd/g dry wt. without EDTA and 500 to 700 μg Cd/g dry wt. in the presence of EDTA. In a second experiment, however, the Cd content in the plants was reduced to negligible levels upon the addition of only 32 μM EDTA. These contradictory results were not explained.

Hardy and O'Keeffe (1985) also examined the effect of EDTA on Cd uptake in E. crassipes. They found that in the presence of 9 μM EDTA, the Cd concentration in the medium dropped only 50% percent after 48 h, instead of the 80% which occurred in the absence of EDTA. The chelators, nitrilotriacetic acid (NTA), N-(2-hydroxyethyl) ethylenedinitrilo-N, N', N'-triacetic acid (HEDTA) and trans-1, 2-cyclohexylene dinitrilotetraacetic acid (CDTA), were also studied. They found that the amount of Cd removed from the medium decreased as the Cd binding constant of the chelator increased.

Unfortunately, Hardy and O'Keeffe (1985) did not measure the uptake of Cd by the plant so it is not known if changes in the external Cd concentration were actually caused by reductions in Cd uptake or by some other phenomenon.

Polar and Kucukcezzar (1986), using L. gibba, found that the effect of EDTA on Cd uptake and toxicity was negligible. Plants were grown in the presence or absence of 27 μM EDTA at up to 27 μM Cd. The internal Cd content at 27 μM Cd was approximately 3,700 ± 1,300 μg Cd/g dry wt. in the absence of EDTA and 2,000 ± 500 μg Cd/g dry wt. in the presence of 27 μM EDTA. There were no consistent differences in the growth rate between cultures grown with or without EDTA. The chelators ethylenediamine-N,N'-bis-(o-hydroxyphenylacetic acid) (EDDHA) and salicylic acid had even less of an effect than EDTA. The results of Polar and Kucukcezzar (1986) must be interpreted with caution, however, because they apparently cultured the plants in a medium containing 50 μM EDDHA. The high chelator concentration in control treatments may have confounded any other chelator effects in their experiments.

The results on the effect of EDTA on Cd uptake and toxicity found in the studies of Nasu et al. (1983), Fayed and Abd-el-Shafy (1985), Hardy and O'Keeffe (1985) and Polar and Kucukcezzar (1986) are inconsistent. In Kwan and Smith (1991), however, the effect of EDTA on Cd uptake in L. minor is consistent. Plants contained 420 μg Cd/g dry wt at 1 μM Cd and 5 μM EDTA. Cadmium uptake was reduced by 75% at 20 μM EDTA and was eliminated at 50 μM EDTA. These results show that EDTA antagonized Cd uptake in the aquatic macrophyte L. minor.

Researchers have also investigated the effect of mineral nutrients on metal uptake and toxicity. Seto et al. (1979) and Kwan and Smith (1991) found that Cd uptake and toxicity increased as the medium was diluted. Kinkade and Erdman (1975)more specifically, studied the effect of water hardness on Cd uptake in Elodea canadensis L. Plants were grown in either soft water with no Ca and Mg or in hard water which contained 150 ppm total of Ca and Mg. After 21 days culture, plants in the hard water

contained ten times less Cd than plants grown in the soft water. The study failed to report the actual external Cd concentration, however, so it is not known if the difference in Cd contents between treatments was due to changes in uptake or by decreases in the external Cd concentration caused by precipitation in the hard water.

Kwan and Smith (1991) examined the effect of Ca on Cd uptake in L. minor. There was a 35% decrease in Cd content as the external Ca concentration was increased from 0 to 160 ppm. Increases above 160 ppm Ca had a negligible effect on Cd absorption. The Ca concentrations in these two studies were considerably higher than in hard-water lakes where the Ca concentration rarely exceeds 80 ppm (Wetzel, 1975). In a study that used lower Ca concentrations, there was no effect of adding 8 ppm Ca on Cd uptake in Eichornia crassipes (Fayed and Abd-El-Shafy, 1985).

Interactions between Zn and Cd have been studied by Hutchinson and Czyrska (1975), O'Keeffe et al. (1984), Hardy and O'Keeffe (1985) and Polar and Kucukcezzar (1986). The latter authors found that increasing the external Cd concentration from 0 to 10 μM decreased the Zn content of L. gibba by approximately 40%. Hardy and O'Keeffe (1985), using E. crassipes, found that the amount of Cd removed from the medium in 24 hours decreased by approximately 80% in the presence of 90 μM Zn. The Zn:Cd molar ratio was 10:1 in this experiment. Zinc was the only ion of nine tested that resulted in a dramatic decrease in Cd removal from the medium. O'Keeffe et al. (1984) also showed that the effect of Zn was dependent on concentration, in that as the Zn concentration increased, the removal of Cd from the medium decreased. Hutchinson and Czyrska (1975) examined the interaction between Zn and Cd uptake and toxicity in L. minor and Salvinia natans (L.) All. at concentrations of 0, 0.75 or 1.25 μM Zn and 0.09 or 0.27 μM Cd. They concluded that increased concentrations of Zn had a synergistic effect on Cd toxicity in these two species. In their experiment, however, there was a stimulatory effect on growth of L. minor and S. natans at even the highest Zn concentration used, indicating that the plants were Zn deficient in all treatments. Under such conditions, interactions

between Cd and Zn may be quite different from results where the plants had an adequate supply of Zn.

In summary, these studies indicate that the uptake and toxicity of metals can be significantly altered by the composition of the medium in which they occur. The most dramatic reduction in the effect of metals occurred when excess chelator was present in the medium. The data suggest that the concentration of EDTA and the chelating capacity of the medium must be defined and controlled in order to produce consistent results on the toxicity of metals. They further suggest that it may be difficult to predict toxicity in the natural environment from external metal concentrations alone.

Cadmium toxicity

Cadmium toxicity data obtained in the laboratory for aquatic macrophytes are variable. As mentioned earlier, Cd EC50 values range from about 0.09 to 1350 µM, depending on the study (Outridge and Noller, 1991). Cadmium toxicity studies can be critically examined on the basis of experimental methods to help understand possible causes of this variability. Based on the preceeding discussion, one of the most important factors which may affect Cd toxicity is the chelating capacity of the medium.

In one of the earliest studies, Stanley (1974) examined the toxicity of a wide range of metals using the rooted submergent Myriophyllum spicatum. Plants were grown in non-axenic static culture in 200 ml tap water for 32 days. The rooting medium consisted of sand or sand mixed with a substance referred to as 'woods earth'. The Cd EC50 for final yield was 65 µM for root dry weight and 130 µM Cd for shoot dry weight. The actual external metal concentrations were not monitored. No indication of the chelating capacity of the test solution was given, though adding soil to the containers likely created conditions favorable for metal chelation. Garvey et al. (1991), for instance, found that soil extracts chelated variable amounts of metals. In Stanley (1974), therefore, there was

no control of the total metal concentration and no definition or control of the chelating capacity and hence the free Cd ion activity. Under these conditions, relating toxicity to a target external Cd concentration is not valid.

The chelating properties of media will also change in non-axenic and/or static cultures. Gipps and Coller (1980), for instance, state that large differences between replicates in metal toxicity are to be expected due to changes in metal speciation and excreted algal metabolites. This problem is exacerbated in static cultures where no renewal of the medium occurs. Wang (1991) found that it was necessary to use a renewal culture method to adequately determine ammonia toxicity to Lemna minor. Hall (1986) showed that Cu toxicity increased four orders of magnitude in the Chlorophytes Chlorella sp. and Chlamydomonas sp. when continuous flow cultures were used instead of static cultures. Replacement of media should occur when duckweeds are cultured to ensure that the toxicant remains at or above 80% of the nominal concentration (U. S. EPA, 1985).

Brown and Rattigan (1979) examined the toxicity of Cd to Elodea canadensis and L. minor. A visual, subjective estimate of plant damage was used to measure toxicity. The medium and substrate were not described so that the chelating capacity of the medium was unknown. Plants were grown with Cd for 28 days in 300 ml of non-axenic medium in static culture at an unknown pH. There was no monitoring of the actual external metal concentration. Whether a soil substrate was included in the L. minor cultures was not indicated. Under these conditions, the EC50 for Cd was 1,300 µM for E. canadensis and 130 µM for L. minor. It is difficult to imagine, however, that this experiment could ever be replicated or that any confidence could be placed in the results given the serious methodological shortcomings outlined above.

Hutchinson and Czyrska (1975) examined Cd toxicity to the floating aquatic macrophytes L. minor and S. natans. As in the studies mentioned above, plants were grown in non-axenic, static cultures. The actual metal concentrations were not monitored and the chelating capacity of the growth medium was unknown. Surprisingly, Cd reduced

the final yield more than 50% at only 0.09 to 0.45 μM external Cd, though it is unknown if this was a significant reduction as no estimates of error were included and no statistical testing was done. This is the lowest reported Cd concentration producing a toxic effect in aquatic macrophytes.

Several other Cd toxicity studies have been carried out using a variety of aquatic macrophytes in static non-axenic cultures with an undefined chelating capacity (Ornes and Wildman, 1979; Van der Werff and Pruyt, 1982; Kay et al., 1984; Polar and Kucukcezzar, 1986; Wang, 1986a, b; Charpentier, 1987). Toxicity estimates in these studies vary between the two extremes reported by Brown and Rattigan (1979) and Hutchinson and Czyrska (1975).

A few studies have used axenic cultures (Nasu and Kugimoto, 1981; Nasu et al., 1983; Nasu et al., 1984; Srivastava and Jaiswal, 1989). Unfortunately, they all contain considerable amounts of glucose. The addition of carbon sources such as glucose, sucrose, yeast extract and bactotryptone is undesirable in toxicity tests as they may complex with the toxicants (ASTM, 1991). This problem is compounded by the fact that media used in these studies were autoclaved. When organic compounds are autoclaved in the presence of mineral nutrients unknown changes occur that can dramatically alter the chelating properties of the medium (pers. obs.). This can easily be demonstrated by autoclaving a Hoagland's medium (Behringer, 1973) with and without sucrose.

In summary, it is evident from the literature that Cd is taken up by, and is toxic to, aquatic macrophytes. Unfortunately, an understanding of the actual concentrations of Cd which are toxic is lacking due to inadequate or inconsistent methodology. Their are several causes for this inconsistency. In none of these studies was there an adequate control or definition of the chelating properties and chemical characteristics of the growth medium. Values of metal toxicity are meaningless if the free ion activity of the metal in the medium is not at least estimated and controlled to some degree. Another problem is that few metal toxicity studies report the measured total metal concentration. Plants grown in

static cultures with a large inoculum and a small volume of medium can very quickly reduce the metal concentration to negligible levels (eg. O'Keeffe et al., 1984; Hardy and O'Keeffe, 1985; Nor and Cheng, 1986). This problem is exacerbated in non-axenic cultures where bacteria, fungi or other plants can also rapidly remove metal contaminants and alter the chelating properties of the medium. Until methodological problems are solved, metal toxicity studies of aquatic macrophytes cannot be interpreted with confidence.

Given these problems, it was interesting to note that amongst the studies where both toxicity and uptake were reported (Hutchinson and Czyrska, 1975; Ornes and Wildman, 1979; Kay et al., 1984; Nasu et al., 1984; Polar and Kucukcezzar, 1986; Charpetier et al., 1987; Srivastava and Jaiswal, 1989), the internal Cd content which occurred at or near the EC50 was more consistent than the external Cd concentration. The internal Cd content at or near the EC50 ranged from approximately 130 µg Cd/g dry wt. in S. natans (Hutchinson and Czyrska, 1975) to 4,300 µg Cd/g dry wt. in L. gibba (Polar and Kucukcezzar, 1986). Most of the data fell between 130 and 1,200 µg Cd/g dry wt. This suggests that when the chelating properties or chemical characteristics of the medium are unknown and/or uncontrolled, the internal metal content is a superior measure of metal availability and hence toxicity than is the external metal concentration. Borgmann et al. (1991) advocate the use of this approach. They found that the toxicity of Cd to the amphipod Hyallela azteca (Saussure), based on the external concentration, varied over two orders of magnitude depending on the characteristics of the water sample. In contrast, the EC50 based on the internal Cd content only varied from 38 to 44 µg Cd/g dry wt. under the same conditions. Toxicity based on the internal metal content, therefore, does not necessarily reflect the chemical characteristics of the external medium.

Objectives

The aim of this thesis is to examine the effects of chemical and biological factors on Cd uptake and toxicity in the submerged aquatic macrophyte Lemna trisulca.

To achieve this, the study is divided into two parts. The first part reports on the development of an effective, defined nutrient medium and the conditions required to successfully and consistently culture the submerged aquatic macrophyte L. trisulca in the laboratory. In this part, Chapter 2 examines requirements for the growth of L. trisulca in axenic culture, and Chapter 3 determines the effect of N, P and Ca on the growth of L. trisulca.

The second part of the study examines Cd toxicity in L. trisulca and the interaction of Cd with biological factors and with Ca, Zn and EDTA. Chapter 4 is a study of the interaction between Ca and Cd. Chapter 5 looks at the interaction between Zn and Cd. Chapter 6 examines the interaction between EDTA and Cd and Zn toxicity and uptake. Chapter 7 determines the response of L. trisulca to Cd over time periods of about 14 days, 6 weeks and 2 years.

Finally, Chapter 8 summarizes the findings on Cd toxicity in L. trisulca.

Chapter 2. Axenic Culture of Lemna trisulca.

INTRODUCTION

Lemna trisulca is an excellent plant for toxicological, physiological and biochemical studies of aquatic macrophytes. It is small in size, grows rapidly, and is easily brought into axenic culture. It also grows entirely submerged, which separates it distinctly from other species of Lemnaceae, and removes complications that might be associated with air/water and water/sediment interfaces (ASTM, 1991).

Despite these advantages, and the fact that the necessary conditions for the laboratory culture of L. trisulca have been outlined by Landolt, 1957 and Bowker et al., 1980, few studies have utilized this plant. This may be because the culture conditions provided by those authors were not conducive to the long-term successful growth of L. trisulca. In particular, Landolt (1957) and Bowker et al. (1980) stated that L. trisulca in axenic culture required organic supplements, acidic pH and low light. More recently, however, Landolt and Kandeler (1987) have suggested that L. trisulca can be grown without organic supplements at higher pH. This chapter critically examines these conditions to determine if L. trisulca can be grown at an alkaline pH using inorganic carbon as the sole carbon source since it has been described as a bicarbonate user (Spence and Maberly, 1985). A second objective was to determine the necessary requirements for the long-term successful culture of L. trisulca and to compare them with culture conditions used to grow duckweeds for toxicity assessment (U. S. EPA, 1985; Hughes et al., 1988: Taraldsen and Norberg-King, 1990; Wang, 1990; ASTM, 1991; Wang, 1991).

MATERIALS AND METHODS

Plants of Lemna trisulca were collected from Delta Marsh (50°11'N, 98°23'W), brought to the laboratory, and rinsed vigorously with several changes of distilled water to remove loosely attached epiphytes. Fronds were placed in a 1.2% sodium hypochlorite / 0.1% sodium dodecyl sulfate solution (Malmberg et al., 1984) for several minutes until they began to turn white. Then they were briefly submersed in 70% ethanol and rinsed several times in sterile water. The survival rate was 1 to 5%. A balance was maintained between exposing plants to the sterilant long enough to kill epiphytes, while at the same time not killing plant meristems. The after-treatment medium (Table 2.1) contained glucose and cultures were discarded if a cloudy suspension developed.

Growth of cultured macrophytes can change over time even under seemingly constant conditions (Huebert and Gorham, 1983; Landolt and Kandeler, 1987; Thorsteinsson et al., 1987; Wang, 1990). In an attempt to prevent seasonal changes in growth, cultures were rotated through an enforced dormancy period. Three cultures of a single clone were alternately kept at 4°C in short days at low light (10 $\mu mol/m^2/s$ photosynthetically active radiation (PAR)) for eight months and at 25°C in continuous, relatively high light (400 $\mu mol/m^2/s$ PAR) for four months. Cultures were used in experiments only during their four month growth period. Stock cultures were subcultured frequently and were grown under similar conditions to those used in subsequent experiments. If the stock cultures became nutrient starved, two to three weeks were required to again produce healthy fronds after transfer of the cultures to fresh media.

Initial culture conditions (Table 2.1) were patterned after those of Landolt (1957) and Bowker et al. (1980). Glucose was added at 55,600 μM at pH 6.5. Cool-white fluorescent light was supplied continuously at 70 $\mu mol/m^2/s$ PAR. The temperature was 25 ± 1°C in the growth chamber and 28 ± 2°C in the flasks. Erlenmeyer flasks fitted with foam stoppers and containing 150 ml of culture medium were sterilized by autoclaving.

Table 2.1. Initial culture conditions used to grow *Lemna trisulca*.

Nutrients	mg/L	µM	Organic Supplement	µg/L
K_2HPO_4	40	230	i-inositol	40
$Ca(NO_3)_2 \cdot 4H_2O$	26	110	D-Ca pantothenate	20
$MgSO_4 7H_2O$	50	203	Folic acid	20
NH_4NO_3	20	250	Thiamin HCl	20
$FeCl_3 \cdot 6H_2O$	2.43	9.0	Riboflavin	20
Na_2NTA[a]	8.46	36.0	Niacin (nicotinamide)	20
$ZnS0_4 \cdot 7H_2O$	0.022	0.08	Pyrodoxine HCl (B_6)	20
$MnCl_2 \cdot 4H_2O$	0.180	0.91	Choline chloride	20
H_3BO_3	0.550	8.87	P-aminobenzoic acid	20
$CuSO_4 \cdot 5H_2O$	0.010	0.04	Biotin	1
$CoCl_2 \cdot 6H_2O$	0.010	0.04	Vitamin B_{12}	1
$Na_2MoO_4 \cdot 2H_2O$	0.170	0.70		
Glucose	10,000	55,600		

Light: 70 µmol/m^2/s PAR continuous cool-white fluorescent

Temperature: 28 ± 2° C

pH: 6.5 ± 0.1

Culture: Three frond inoculum in 150 ml batch culture, 28 days incubation

a. Disodium Nitrilotriacetic acid

Vitamins were filter-sterilized and added later. Cultures were not aerated. The inoculum was a three frond fragment and was grown for 28 days in batch culture.

Plants grew well under these initial conditions for 5 to 7 days, but then languished and eventually died. In an attempt to improve growth, modifications to the initial culture conditions were made in a series of twelve experiments. Successful modifications were retained and used in subsequent experiments (Table 2.2, Exp. 1-12).

The reduction of glucose from 55,600 to 1,110 μM ensured the survival of plants in Exp. 1. Cultures were aerated in Exp. 2. In Exp. 3, the culture medium was replaced once per week over the 28 day growth period, and the pH increased from 6.5 to 7.8 by adding sodium bicarbonate. In Exp. 4, light was increased from 70 to 400 $\mu mol/m^2/s$ PAR. Glucose was removed from the culture medium in Exp. 5.

Datko et al. (1978) outlined a method for the semi-continuous culture of Lemnaceae. In a modification of this technique (Exp. 6), 750 ml of medium in 1-L flasks was used and replacement medium was delivered once daily at a rate of approximately 6 ml/frond/doubling time.

Autoclaving may produce unknown precipitates and alter organic compounds. Filter sterilization with 0.2 μm membrane filters was thus adopted after Exp. 6. Experiment 7 tested the effect of the sulfonic acid buffer N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) in filtered media. In Exp. 8, ammonia was removed from the medium and in Exp. 9, nitrilotriacetic acid (NTA) was replaced by ethylenediaminetetraacetic acid (EDTA) at a 1:1 molar ratio with Fe. In Exp. 10, vitamins were removed from culture media and in Exp. 11 the micronutrients were doubled. Finally, in Exp. 12, the inoculum was reduced from three fronds to one frond.

Measured parameters included multiplication rate (MR) and doubling time (DT) in days based on frond production, final yield in mg dry wt. and individual frond biomass (μg/frond). For multiplication rate;

$$MR = 1000(\log F_t - \log F_o)/t \qquad \text{Eq. 1}$$

where F_t is the final frond number, F_o is the initial number of fronds and t is the growth period in days. The constant '1,000' is included to make the MR a three digit whole number and, though not entirely necessary, is often used when examining growth rates in Lemnaceae (Hillman, 1961; Landolt and Kandeler, 1987). DT = 301/MR. To determine dry weight, plant material was oven-dried at 85°C for 24 h. Three replicates per treatment were used in all experiments and data are represented as mean ± one standard deviation. The data from these experiments are contained in Table A.1 of Appendix A. This chapter has been published as Huebert et al. (1990).

RESULTS AND DISCUSSION

Cultures containing 55,600 μM glucose (Table 2.1) routinely died even though the initial MR was high. The sharp reduction in pH to 3.8 as glucose was taken up during the experiment may have caused this death. Hillman (1961) observed this phenomenon which may occur because glucose transport is most likely by proton symport (Reinhold and Kaplan, 1984). With this mechanism, a low pH increases the efficiency of glucose transport.

Reducing the glucose level ensured survival of the plants but produced very poor growth (Table 2.2, Exp. 1) with a DT of almost 9 days, a MR of 34, and final yield of only 5 mg dry wt. in 28 days. This is much lower than the maximum DT of 3.0 days reported by Landolt (1957).

Frond biomass was calculated for all treatments, but there were no consistent trends in these data. Excluding one high value of 551 μg/frond, the average biomass was 270 μg/frond and ranged from 234 to 305 μg/frond after 14 days growth (Table 2.2).

Aeration of L. trisulca cultures produced a 40% reduction in DT to 5.3 days and a concomitant increase in MR to 57 (Table 2.2, Exp. 2). The final yield increased more than eight-fold to 43 mg dry wt. Plants in aerated cultures also appeared much healthier.

Table 2.2. Doubling time (DT) in days, multiplication rate (MR), final yield (mg dry wt.) and frond biomass (μg/frond) of Lemna trisulca cultures grown under various conditions for 14 or 28 days. Values are mean ± sd, n = 3.

	Experiment[a]	DT	MR	Final yield	Frond weight
1	1,110 μM glucose	8.9 ± 0.9	34 ± 3	5 ± 1	294 ± 84
2	Aeration	5.3 ± 0.1	57 ± 1	43 ± 4	551 ± 27
3	Replacement + pH 7.8	4.1 ± 0.1	74 ± 3	67 ± 4	281 ± 40
4	400 μmol/m^2/s PAR	4.0 ± 0.2	76 ± 3	81 ± 10	305 ± 30
5	No glucose	4.2 ± 0.1	71 ± 2	69 ± 6	240 ± 6
6	Exponential replacement	3.8 ± 0.1	80 ± 1	122 ± 7	234 ± 9
7	Filter sterilize + HEPES[b]	-------	--------	-------	-------
8	Filter sterilize - NH_4	3.5 ± 0.2	86 ± 5	238 ± 64	304 ± 38
9	EDTA[c]	1.9 ± 0.1	157 ± 3	128 ± 15	267 ± 5
10	No vitamins[c]	2.0 ± 0.1	149 ± 5	104 ± 10	284 ± 31
11	[c]2X micronutrients	1.9 ± 0.1	162 ± 1	135 ± 2	246 ± 9
12	[c]1 frond inoculum	1.7 ± 0.1	179 ± 5	84 ± 11	261 ± 7

a. Successful modifications were retained and used in subsequent experiments.

b. N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

c. Values are for cultures after 14 days instead of 28 days.

Replacing the nutrient medium totally once per week, or increasing the pH to 7.8, had no effect on growth (data not shown). These two factors in combination, however, resulted in a DT of 4.1 days, a decrease of approximately 23%. The MR rose to 74 and the final yield increased over 50% to 67 mg dry wt. in 28 day cultures (Table 2.2, Exp. 3). Clearly, high glucose levels (organic carbon) and low pH are not essential for the growth of L. trisulca. These results differ from those of Landolt (1957) and Bowker et al. (1980).

Increasing the light intensity from 70 to 400 $\mu mol/m^2/s$ PAR promoted growth slightly (Table 2.2, Exp. 4). Final yield increased from 67 to 81 mg dry wt., the MR increased to 76, and the DT dropped to 4.0 days. Photo-damage in the form of bleaching was not evident at the higher light intensity, and all fronds appeared green and healthy.

The complete removal of glucose from 150 ml batch cultures produced excellent growth initially, with a DT of 3.0 days in the first 14 days, but fronds later became stunted, chlorotic, and misshapen. This may have been caused by nutrient limitation, since autoclaving this alkaline medium produced considerable precipitate. The final DT was 4.2 days with a drop in final yield to 69 mg dry wt. (Table 2.2, Exp. 5).

Larger solution volumes and a semi-continuous, exponential replacement rate eliminated this problem. Plants in cultures lacking glucose grew well with a DT of 3.8 days, a MR of 80, and a final yield of 122 mg dry wt. in 28 days (Table 2.2, Exp. 6). Fronds looked green and healthy.

Glucose is therefore not an essential requirement for the culture of L. trisulca. Inorganic carbon alone supported excellent growth when supplied at 500 μM $NaHCO_3$. This is reasonable since L. trisulca has been labelled a bicarbonate user (Spence and Maberley, 1985) and is in agreement with Landolt and Kandeler (1987), who suggested that L. trisulca could be grown without glucose at higher pH levels.

The buffer HEPES was acutely toxic to L. trisulca at 5 mM (Table 2.2, Exp. 7). Bowker et al. (1980) also found TRIS and phosphate buffers to be unsuitable for

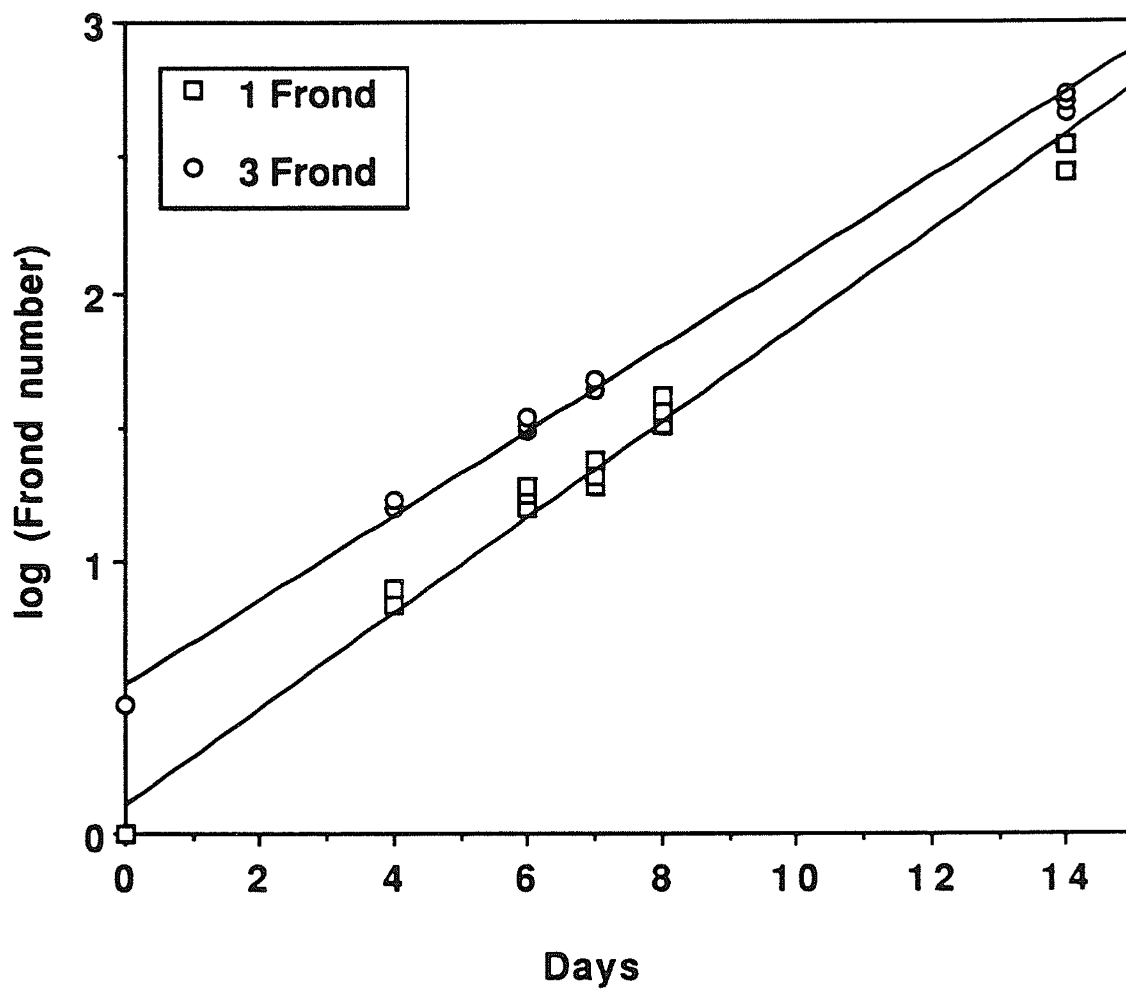
culturing. Buffers were therefore not used in L. trisulca cultures. Ammonia was removed in order to maintain an alkaline pH without buffers. This did not adversely affect growth (Table 2.2, Exp. 8). With nitrate alone, the DT was 3.5 days, the MR was 86 and the final yield was 238 mg dry wt. in 28 days.

The use of EDTA as an Fe chelator at a molar ratio with Fe of 1:1 (Chapter 6) instead of NTA at a 4:1 molar ratio with Fe greatly improved growth, suggesting NTA toxicity in filtered media. The DT dropped to 1.9 days, the MR increased to 157, and the final yield was 128 mg dry wt. in 14 days. The growth period was reduced from 28 to 14 days because of the rapid growth under these conditions (Table 2.2, Exp. 9). The absence of a buffer and the presence of nitrate alone caused a gradual rise in pH to between 8.1 and 8.2 after 12 days and then a further increase to between 8.4 and 8.8 in the final 2 days.

The removal of vitamins from the culture medium had little effect on growth as evidenced by a DT of 2.0 days, a MR of 149 and a final yield of 104 mg dry wt. in 14 days (Table 2.2, Exp. 10). The suggestion (Landolt, 1957; Bowker et al., 1980) that organic supplements or vitamins are essential for L. trisulca growth was not supported by my results. Doubling the micronutrients also had little effect on the multiplication rate, and therefore were maintained at original levels in subsequent experiments (Table 2.2, Exp. 11).

Reducing the inoculum from three fronds to one increased the multiplication rate from 157 to 177 and decreased the DT from 1.9 to 1.7 days (Table 2.2, Exp. 12; Fig. 2.1). This may have been because of crowding in the flasks with a three frond inoculum but the anatomy of L. trisulca may also have contributed to the rise in MR with a one frond inoculum. This is because a three frond inoculum actually has four meristems and a one frond inoculum has two, so that the latter is half as large as the former and not a third. Calculated on the basis of meristems, the MR was 158 for a one frond inoculum and 150 for a three frond inoculum.

Figure 2.1. The growth of Lemna trisulca over 14 days where $\log F_t$ is plotted against t. For the one frond inoculum, $y = 0.1 + 0.177x$, $R^2 = 0.99$, and for the three frond inoculum, $y = 0.5 + 157x$, $R^2 = 0.99$.



1 Frond
3 Frond
log (Frond number)
Days
0
1
2
3
0
2
4
6
8
10
12
14

Equation 1 can be rearranged to ;

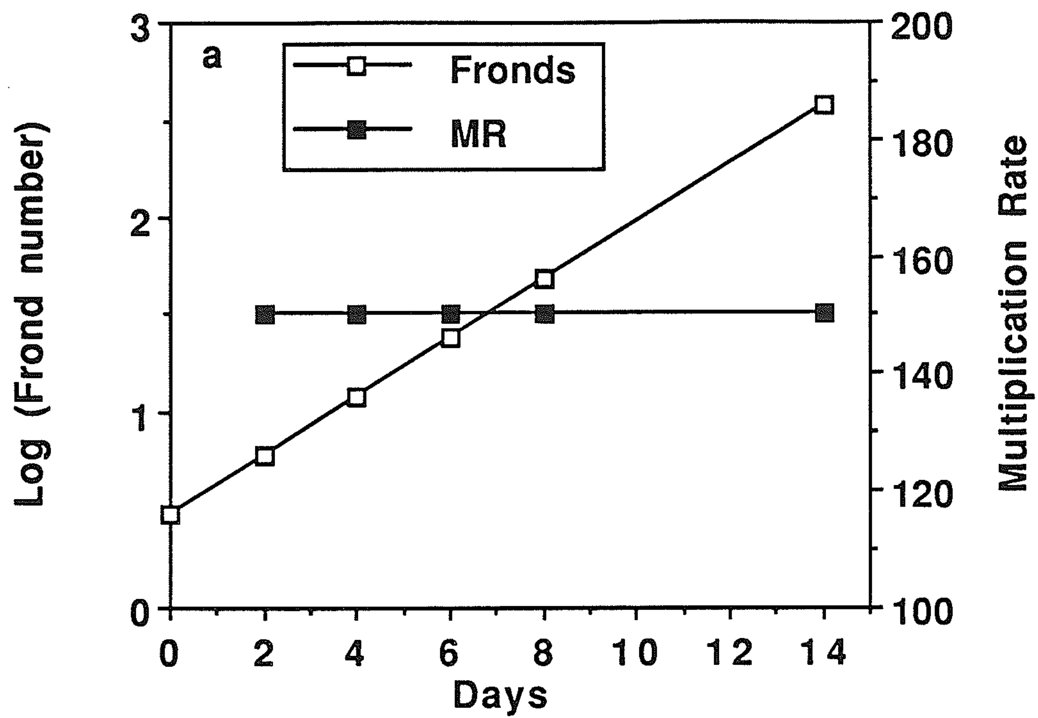
$$\log F_t = \log F_o + MR * t \qquad \text{Eq. 2}$$

which indicates there is a linear relationship between $\log F_t$ and t over 14 days when growth is exponential (Fig. 2.2a). The MR is the slope of the line and is constant over time (Fig. 2.2a). The final culture conditions used to grow L. trisulca (Table 2.3) are radically different from those proposed by Landolt (1957) and Bowker et al. (1980), who stated that organic supplements, acidic pH and low light conditions were required.

In comparison, the inocula sizes and volumes of test solution in the studies that use duckweeds for toxicity assessment (U. S. EPA, 1985; Hughes et al., 1988: Taraldsen and Norberg-King, 1990; Wang, 1990; ASTM, 1991; Wang, 1991) range from 4 fronds in 150 ml (U. S. EPA, 1985) to 20 fronds in 15 ml (Wang, 1990, 1991). Only two studies (U. S. EPA, 1985; Taraldsen and Norberg-King, 1990) include replacement of media in their protocol. Under the conditions of the above studies, F_t plotted against t is linear (Hughes, 1988; Wang, 1990, 1991), which means that the number of fronds produced per day is constant (Fig. 2.2b) and the MR decreases exponentially over time. In Figure 2.2b, the MR decreased from 301 to 32 over 14 days when the increase in frond number was a constant 15 frond/day (see Wang, 1990). This indicates that the plants are under stress and suggests that the amount of plant material used to inoculate the cultures is too large for the volume and replacement rate of the media. When growth is linear, studies using duckweeds will be confounded by the relative decrease in the MR of control cultures compared with treatments and by nutrient stress in the plants.

If meaningful data are to be generated in toxicological studies, the volume of media, replacement schedule and inoculum size must be designed to ensure that exponential growth occurs throughout the test period and that, consequently, the MR is constant. This has been accomplished for L. trisulca using the conditions listed in Table 2.3.

Figure 2.2. The relationship between the increase in the number of fronds over time and the multiplication rate. Figure 2.2a describes an exponential increase in frond number where $\log F_t$ is plotted against time. Fig. 2.2b describes a linear increase in frond number where F_t is plotted against time. F_t is the final number of fronds.


a
Fronds
MR
Log (Frond number)
Multiplication Rate
Days
0
1
2
3
100
120
140
160
180
200
0
2
4
6
8
10
12
14

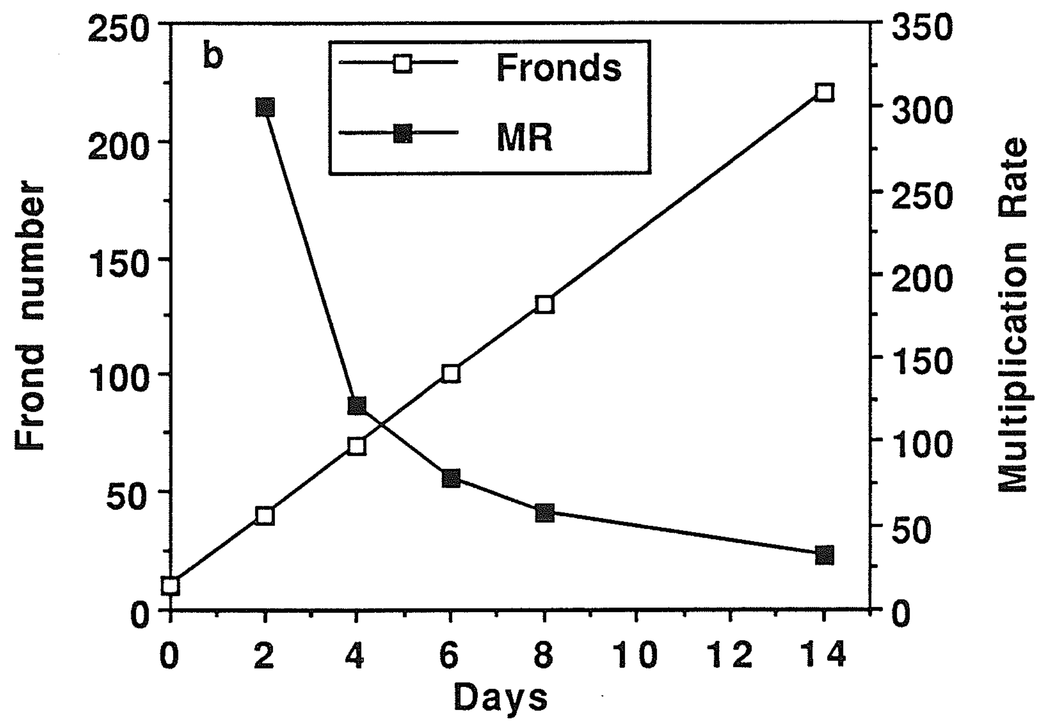

b
Fronds
MR
Frond number
Multiplication Rate
Days
0
50
100
150
200
250
0
50
100
150
200
250
300
350
0
2
4
6
8
10
12
14

Table 2.3. Final culture conditions for Lemna trisulca

Nutrient[a]	mg/L	μM	Micronutrient[a]	mg/L	μM
KNO_3	50.5	500	$ZnSO_4 \cdot 7H_2O$	0.022	0.08
$CaCl_2 \cdot 2H_2O$	16.2	110	H_3BO_3	0.550	8.87
$MgSO_4 \cdot 7H_2O$	50.0	203	$CuSO_4 \cdot 5H_2O$	0.010	0.04
Na_2HPO_4	34.1	240	$CoCl_2 \cdot 6H_2O$	0.010	0.04
$FeCl_3 \cdot 6H_2O$	2.43	9.0	$Na_2MoO_4 \cdot 2H_2O$	0.170	0.70
$Na_2EDTA \cdot 2H_20$[b]	3.35	9.0	$MnCl_2 \cdot 4H_2O$	0.180	0.91
$NaHCO_3$	42.0	500			

Light: 400 $\mu mol/m^2/s$ PAR continuous cool-white fluorescent

Temperature: 28 ± 2° C

pH: 7.8 ± 0.1

Culture: One or three frond inoculum in 750 ml axenic, filter-sterilized, aerated culture (125 ± 15 ml/min) with exponentially increasing replacement of media and 14 days culture

a. All chemicals of ACS grade. Glassware was acid washed in 3N HNO_3 and rinsed with deionized water (specific conductivity of 0.056 ± 0.002 μS/cm).

b. Disodium ethylenediaminetetraacetic acid

Chapter 3. Phosphorus, nitrogen and calcium effects on Lemna trisulca

INTRODUCTION

Lemna trisulca obtains its nutrients entirely from the water column. This growth habit is unlike most other submerged macrophytes, which can satisfy many of their nutritional requirements, particularly for N and P, from the sediment via root uptake (Bole and Allen, 1978; Carignan and Kalff, 1980; Barko and Smart, 1981; Huebert and Gorham, 1983). It is therefore important to understand the ability of L. trisulca to absorb water column N and P, since it is much more likely to be limited by these nutrients than are rooted aquatic plants.

Few studies have examined the characteristics of nutrient uptake and limitation in submerged macrophytes over a wide range of external concentrations. Gerloff and Krombholz (1966) studied the effect of varying the external P and N supply in batch culture of Vallisneria americana Michx. They found that the P content in the plant decreased as the external concentration decreased but that growth was unaffected until the internal P content fell to 0.13% dry wt. Growth was proportional to internal P content below this value under the specific conditions of their study. Similarly, a critical concentration was determined for N of 1.3% dry wt. Mickle (1975), using the same culture techniques, determined a critical concentration for P of 0.10% dry wt. for Ceratophyllum demersum and 0.07% dry wt. for Myriophyllum spicatum. More recently, Van Wijk (1989) studied P and N uptake in Potamogeton pectinatus L. using a flow through culture method. A critical internal P content of 0.15% dry wt. was estimated, which occurred at an external P concentration of 3.3 μM. A comparable value for N was not determined since growth was limited at even the highest external N level. Reddy et al. (1989) examined N uptake in floating Eichornia crassipes and related biomass production in response to loading rate. The lowest internal N content was 0.8% dry wt. at the minimum loading rate.

There is less experimental work quantifying the response of macrophytes to Ca. Demarte and Hartman (1974) found that Ca was taken up by both roots and shoots. Huebert and Gorham (1983) determined that Ca in the water column was essential for the survival of P. pectinatus, especially in the presence of other divalent cations. They noted that excellent growth was supported by 50 μM Ca, which is well below the Ca concentration normally associated with the presence of P. pectinatus in nature. Gerloff (in Mickle, 1975) determined a critical Ca content of 0.22% dry wt. in C. demersum.

The purpose of this study was to examine the morphology and multiplication rate of L. trisulca in relation to external N, P and Ca concentrations. This was a preliminary study in the determination of the effects of P and Ca on Cd uptake and toxicity in L. trisulca.

MATERIALS AND METHODS

Axenic stock cultures of Lemna trisulca were maintained in 150 ml of autoclaved medium (Table 2.3) within 250 ml Erlenmeyer flasks. Material was grown exponentially for 5 to 7 days before being used in experiments. For experiments, a three-frond fragment of L. trisulca was placed into a 1-L Erlenmeyer flask containing 750 ml of filter-sterilized medium (Table 2.3). The cultures were aerated with ambient air passed through a humidifier containing deionized water, a cotton filter and a 0.45 μ sterile filter. Light was supplied continuously at 400 μmol/m^2/s PAR by Sylvania cool-white fluorescent fixtures. The temperature was 25 ± 1°C in the growth chamber and 28 ± 2°C in the experimental flasks. Replacement of media occurred once or twice daily, increasing exponentially at a rate of 6 ml/frond/doubling time based on a 2 days doubling time.

Plants were grown for 14 days at the specified N, P or Ca level. For the P experiment, nutrients were as described in Table 2.3 with the further addition of vitamin supplements (Bowker et al., 1980) which were discarded in later experiments (Chapter

2). The external P concentrations were set at 240, 120, 60, 30, 15, 7.5, 3.8, 1.9, 0.94, 0.47, 0.23 or 0 µM. For the N experiment, P was set at 7.5 µM and the initial external N concentrations were set at 500, 125, 31, 7.8, 1.9 or 0 µM. For the Ca experiment, P was set at 15 µM, N at 250 µM and the external Ca concentrations were set at 880, 440, 220, 110, 27.5, 6.9, 1.7 or 0 µM. These concentrations were chosen to cover the range from nutrient excess to limitation.

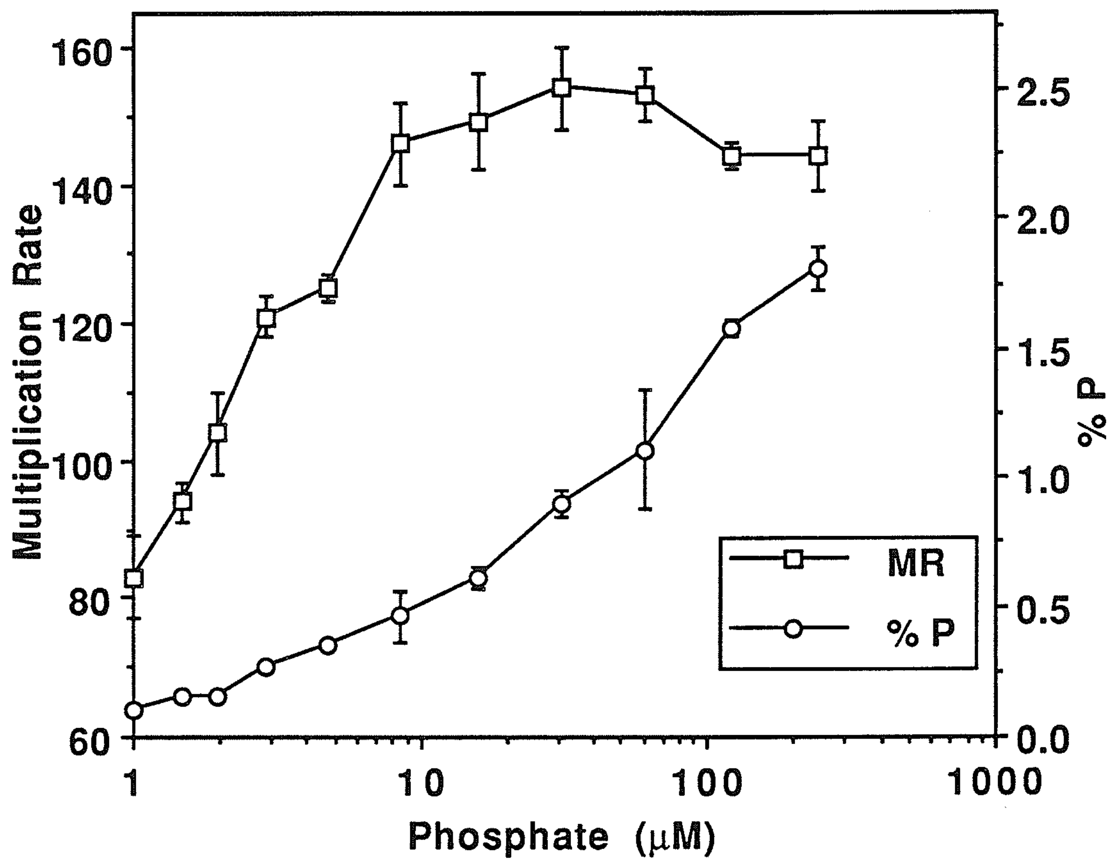
Plants were removed from the culture flasks after 14 days and the fronds counted. A frond was tallied if the root bud was visible below the blade of the frond. Plants were washed three times in distilled water and dried for 24 hours at 85°C in 40 ml ceramic crucibles to determine dry weight. Concentrated HNO_3 was added and heated to dryness after which the crucibles were heated at 450°C for 6 h. Samples were dissolved in heated 5N HCl, filtered with Whatman #42 ash-free filter papers and diluted to 100 ml with deionized water. Phosphate was determined by the molybdate blue method (Stainton et al., 1977) and Ca by atomic absorption spectroscopy. All reagents were ACS grade or better. Glassware was acid washed in 3N HNO_3 and rinsed repeatedly with deionized water. The water used throughout was distilled, deionized and had a final conductivity of approximately 0.06 µS/cm. Three replicates were used for each treatment and all data are presented as a mean ± standard deviation. The data from this chapter are contained in Tables A.2.1,2 and 3 in Appendix A. This chapter has been published as Huebert and Shay (1991a).

RESULTS AND DISCUSSION

Phosphorus

In cultures exposed to external P concentrations of 240 to 7.5 µM, the MR averaged 148 ± 6 and the final yield averaged 87 ± 11 mg dry wt., while the internal P concentration declined from 1.8 to 0.46% dry wt. (Table 3.1, Fig. 3.1). The MR and

Figure 3.1. Multiplication rate (MR) and internal phosphorus (as % dry wt.) for Lemna trisulca in response to external phosphate (log[1 + μM PO_4]).



Multiplication Rate
160
140
120
100
80
60
% P
2.5
2.0
1.5
1.0
0.5
0.0
1
10
100
1000
Phosphate (μM)
MR
% P

Table 3.1. The effects of external P, N and Ca on final yield (mg dry wt.) after 14 days. Data are presented as means ± standard deviation.

P		N		Ca	
µM	Yield	µM	Yield	µM	Yield
240	73 ± 8	500	56 ± 8	880	34 ± 15
120	73 ± 2	125	55 ± 4	440	41 ± 1
60	94 ± 12	31	49 ± 10	220	88 ± 9
30	99 ± 18	8	24 ± 5	110	81 ± 10
15	88 ± 26	2	14 ± 1	27.5	69 ± 8
7.5	95 ± 12	0	9 ± 2	6.9	12 ± 1
3.8	53 ± 3			1.7	-------
1.9	46 ± 8			0	-------
0.9	28 ± 5				
0.4	20 ± 2				
0	17 ± 4				

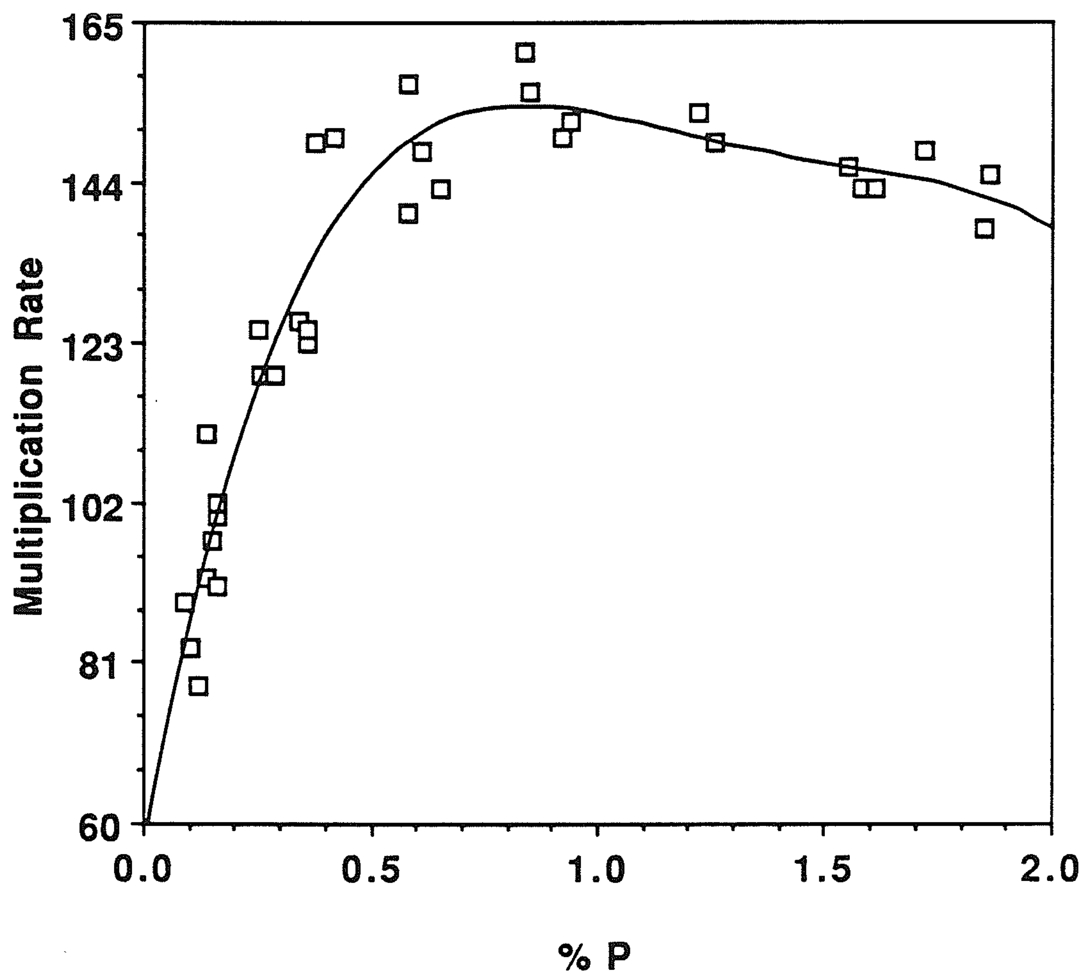
final yield began to decrease when the initial external P concentration was lowered below 7.5 µM. Plants grown without external P had a MR of 83, a final yield of 17 ± 4 mg dry wt. and an internal P concentration of 0.10% dry wt. (Table 3.1, Fig. 3.1). The P in the L. trisulca inoculum approximately accounted for the P in the plant at the end of this treatment and was sufficient for four frond doublings in 14 days. As the external P and the MR increased, this initial P pool became less important, accounting for less than 5% of total internal P above external P of 3.8 µM.

The inputs, outputs and plant uptake of P were used to estimate the P in the media at any given time during the experiments (Datko et al., 1978). Estimates were checked by actual monitoring in the experiment run at 15 µM initial external P and agreed, on average, to within two percentage points. As the initial external P concentration was decreased, the proportion of the total available P sequestered by the plants in the 14 day growth period increased from 4.4% at 240 µM to 44% at 7.5 µM. The P concentration in the medium decreased to approximately 4.2 µM by the end of this latter treatment. Below 7.5 µM this proportion fluctuated but did not increase further. These data suggest that 4.2 to 7.5 µM was a threshold concentration at which the efficiency of P uptake from the medium was maximized.

The relationship between internal P content and MR was described by a fourth order polynomial (Fig. 3.2). The inflection point suggests that in L. trisulca the critical P content under these specific conditions was about 0.65% dry wt. which occurred at an external P concentration of between 7.5 and 15 µM. The individual data points, however, suggest that the MR began to decrease rapidly below 0.46% dry wt. internal P which occurred at a P level of 4.2 to 7.5 µM (Fig. 3.2). The variability in the data at this critical point make precise estimates difficult. Either of these estimates is high compared with those of Gerloff and Krombholz (1966), Mickle (1975) and Van Wijk

Figure 3.2. Multiplication rate vs internal phosphorus (as % dry wt.) in Lemna trisulca.

$y = 56 + 328x - 391x^2 + 195x^3 - 36x^4$, $R^2 = 0.93$.



165
144
123
102
81
60
Multiplication Rate
0.0
0.5
1.0
1.5
2.0
% P

(1989). The difference can be ascribed to the fact that all these authors measured biomass and not growth rate. If the growth rate was not constant over time in these studies, different initial rates could produce similar biomass values. In addition, to determe growth in batch culture (Gerloff and Krombholz, 1966; Mickle, 1975) when demand exceeds supply, reflects the plant's ability to dilute its internal reserves. The critical point occurs when the plant can no longer dilute internal P to increase its biomass. Gerloff and Krombholz (1966) and Mickle (1975) determined this to be between 0.07 and 0.15% dry wt. P. To determine relative growth rate in semi-continuous culture at a constant growth rate, on the other hand, measures the ability of the plant to sequester P at a constant external concentration to meet the demands for that rate of growth. The critical point occurs when the kinetics of uptake under these conditions can no longer supply enough P to maintain a maximum rate of growth. This occurred between 4.2 and 15 µM external and 0.46 and 0.65% dry wt. internal P in L. trisulca. The results of this method should be close to that of Gerloff and Krombholz (1966) when the demand for P even at a low rate of growth greatly exceeds the external supply rate. The internal P at these levels corresponded closely at 0.10% dry wt. (Fig 3.2) to the critical concentration calculated by Gerloff and Krombholz (1966). The external values cited above should not be taken as representative of limiting levels in a natural situation since it is known that the Lemnaceae possess phosphatases and metabolize organic phosphates to supplement often limiting inorganic supplies (Landolt and Kandeler, 1987).

The L. trisulca plants maintained a green and healthy appearance at even the lowest P concentration. However, at initial external P concentrations of 7.5 µM or less, the frond weight increased, initially to 280 µg/frond at 7.5 µM, then as high as 389 µg/frond (Table 3.2). The root length also responded to decreased P levels. Average root length was 24 ± 3 mm (n = 12) at 240 µM P and increased to 42 ± 4 mm (n = 12) at 3.8 µM P (Table 3.2).

Table 3.2. Frond weight (μg/frond), root length (mm), and % of total available P used by Lemna trisulca in response to external P concentration. Data are presented as a mean ± standard deviation.

	Phosphate (μM)						
Parameter	240	60	15	7.5	3.8	0.9	0
frond weight	235 ± 13	229 ± 15	232 ± 19	280 ± 19	314 ± 20	323 ± 9	389 ± 34
root length	24 ± 3	36 ± 4	40 ± 4		42 ± 4		
% used	4.4 ± 4	14 ± 1	28 ± 7	44 ± 7	37 ± 1	25 ± 4	

Nitrogen

The MR averaged 129 ± 3 and final yield averaged 53 ± 8 mg dry wt. as the external N concentration was lowered from 500 to 31 μM (Table 3.1, Fig. 3.3). The latter N concentration is lower than the 57 μM N that Van Wijk (1989) found to be limiting for P. pectinatus. Below 31 μM, the MR declined steadily and reached a minimum of 61 when no N was added (Fig. 3.3). The N in the inoculum was sufficient for approximately two doublings and produced 9 ± 2 mg dry wt. when the L. trisulca stock cultures were grown at 500 μM N. Fronds of L. trisulca appeared well-formed at even the lowest level of N, though they became pale and translucent.

Calcium

At Ca concentrations between 27.5 and 220 μM the MR averaged 143 ± 6 and final yield averaged 80 ± 11 mg dry wt. The MR dropped to 121 at 440 μM and 113 at 880 μM external Ca (Table 3.1, Fig. 3.4). The internal Ca content increased from 0.83 to 3.29% dry wt. within this same range (Fig. 3.4 and 3.5). The inhibition of growth at only 440 μM Ca (17.6 ppm) was perhaps not due to Ca toxicity, but to the 15 μM P needed to culture L. trisulca effectively. This is considerably higher than the level of total P found in even hypereutrophic lakes (Wetzel, 1975). Precipitation of calcium phosphate is likely under these conditions, causing P shortages, especially at alkaline pH values (Wetzel, 1975).

The MR decreased abruptly below 27.5 μM Ca, dropping to 51 at 6.9 μM Ca and to 0 at 1.7 μM Ca (Fig. 3.4). The MR measured at 6.9 μM was only a rough estimate since the fronds, even though still green, became small, twisted and difficult to count. The final yield at this concentration was 12 ± 1 mg dry wt. The sharp decline in MR and change in morphology at low Ca concentrations contrasts with the effect of decreasing the external P and N levels where morphology was not affected. The decline in MR corresponded with a small decrease in the internal Ca content from 0.83 to 0.55% dry wt.

(Fig. 3.5). These values are somewhat lower than the mean of 1.77% dry wt. calculated for aquatic macrophytes by Hutchinson, (1975) but well above the critical Ca content of 0.22% dry wt. calculated by Gerloff (in Mickle, 1975).

Calcium has also been implicated as a nutrient important outside the symplasm in plants. It is essential for cell wall development, cell membrane integrity, and appears to be required in the free space surrounding the cell membrane which acts as a reservoir for transient Ca pulses into the cell (Hepler and Wayne, 1985; Kauss, 1987). The data suggest that the external concentration of Ca is more important than the internal concentration for controlling MR and morphology.

Figure 3.3. The multiplication rate of Lemna trisulca in response to external nitrogen (log[1 + μM N]).

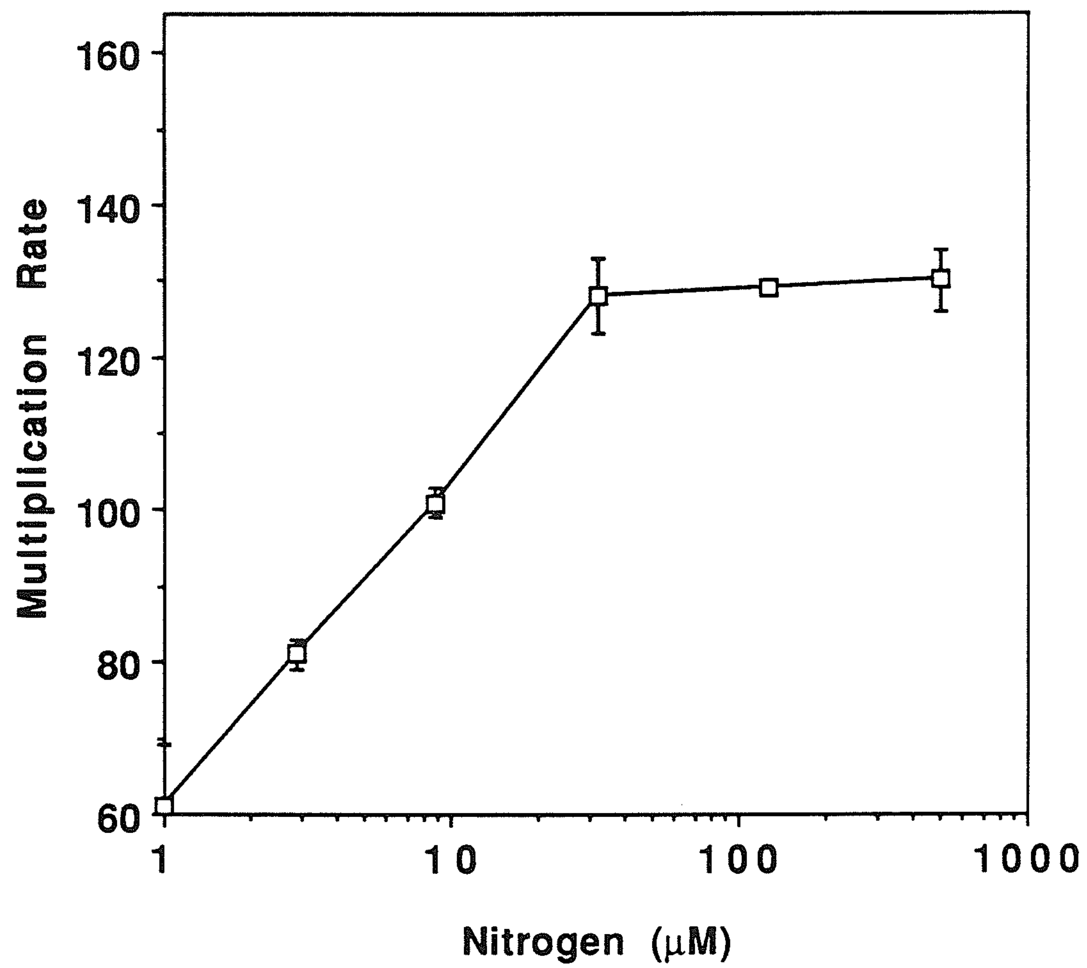
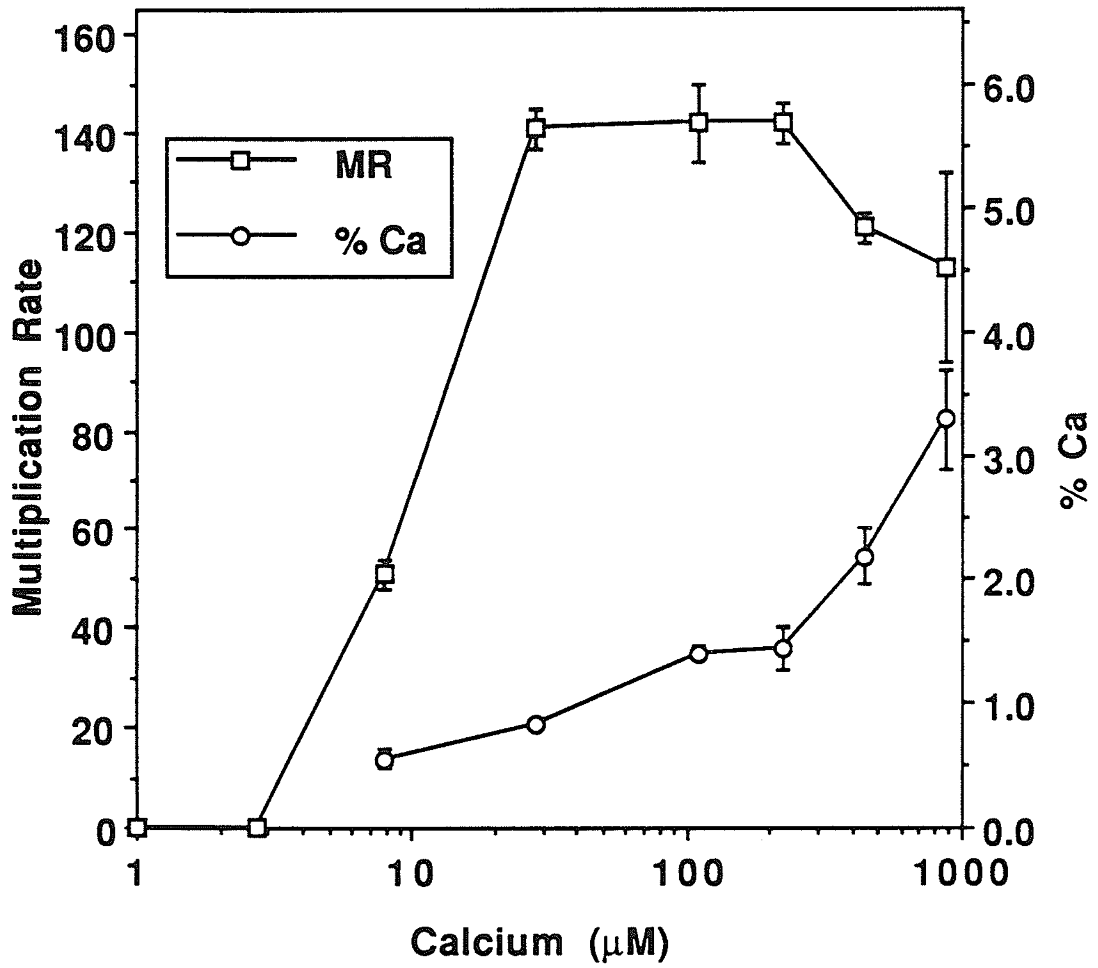
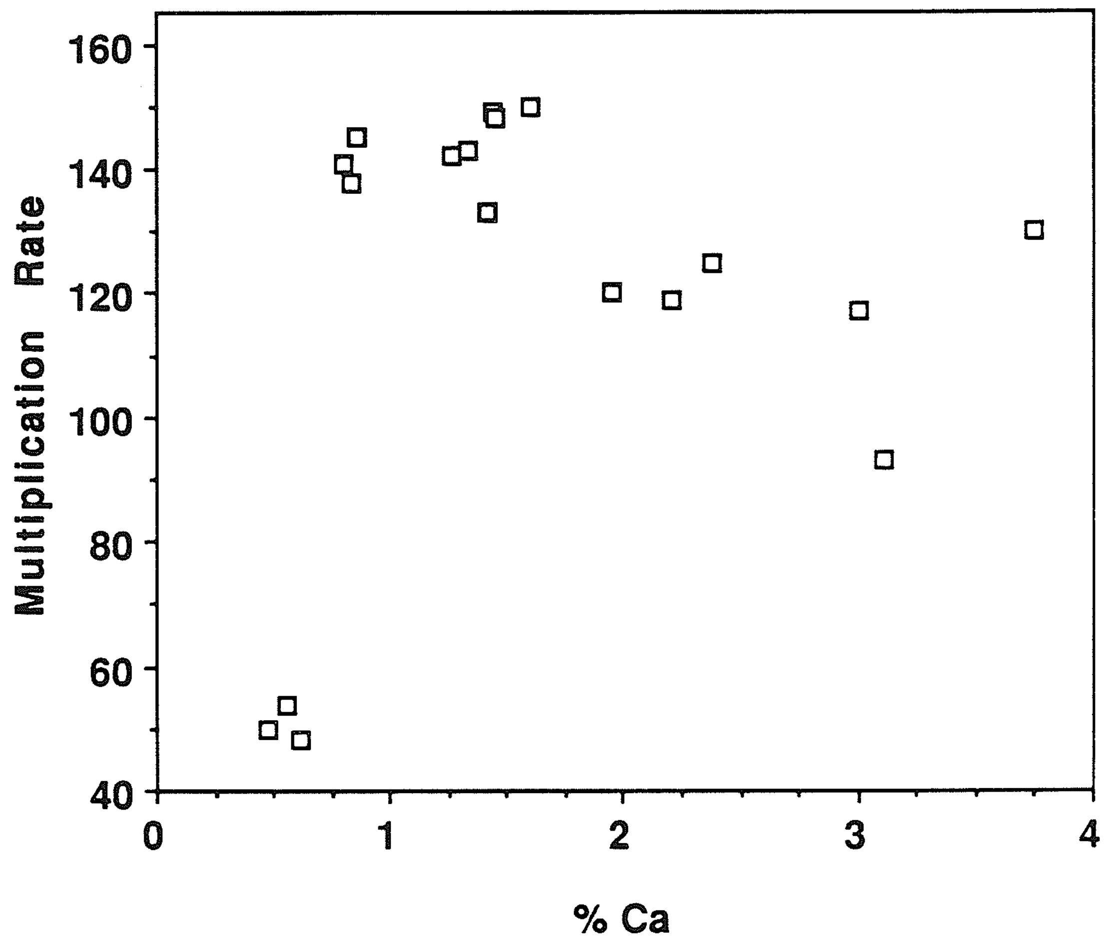

Multiplication Rate
160
140
120
100
80
60
1
10
100
1000
Nitrogen (µM)

Figure 3.4. The multiplication rate and % internal calcium of Lemna trisulca in response to external calcium (log[1 + μM Ca]).



MR
% Ca
Multiplication Rate
% Ca
Calcium (μM)
160
140
120
100
80
60
40
20
0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1
10
100
1000

Figure 3.5. The multiplication rate vs internal calcium (as % dry wt.) in Lemna trisulca. The data are from treatments containing between 27.5 and 880 μM external calcium.


Multiplication Rate
160
140
120
100
80
60
40
0
1
2
3
4
% Ca

Chapter 4. Cadmium toxicity and its interaction with calcium.

INTRODUCTION

Cadmium is a rare but highly toxic heavy metal which is used in pigments and batteries, for electroplating, and as a plastic stabilizer, processes which largely preclude its reuse (Nriagu, 1980). The resultant redistribution of Cd into the aquatic environment occurs through sewage and industrial effluent (Forstner and Wittman, 1983).

Hutchinson and Czyrska (1975) found Cd toxic to Lemna minor and Salvinia natans at 0.45 and 0.09 μM, respectively. Wang (1986a) also found significant decreases in growth of L. minor at 0.45 μM Cd. Other workers have reported toxic effects at 1.0 μM in L. paucicostata (Hegelm.) (Nasu et al., 1983), 1.8 μM in Spirodela polyrhiza (Charpentier et al., 1987), 4.45 μM in Eichornia crassipes (Kay et al., 1984) and around 9.0 μM in L. gibba L. (Polar and Kucukcezzar, 1986) and S. polyrhiza L. (Srivastava and Jaiswal, 1990). These differences may be ascribed to the species used, the response parameter tested and perhaps to the medium since it is known that mineral nutrients (Seto et al., 1979; Nasu et al., 1984) and especially chelators can affect the toxicity of heavy metals to higher plants (Schreinemakers and Dorhout, 1985; Polar and Kucukcezzar, 1986) and algae (Thompson and Couture, 1990). In addition, all the above studies were carried out using batch cultures and only two were based on axenic culture techniques. Since toxicity of heavy metals is thought to be related to the activity of the ion and not to the total concentration (Borgmann, 1983), it is possible that the effects were confounded by changes in the nutrient media over time and by unknown and variable levels of other organisms. This could partly explain the differences in the Cd concentrations required to cause a noticeable effect.

Calcium is an essential plant nutrient needed for cell wall development and cell membrane integrity. It accumulates in the apoplasm and is used for transient Ca pulses

into the cell (Hepler and Wayne, 1985; Kauss, 1987). In submerged aquatic macrophytes this apoplastic Ca pool can interact with the external nutrient medium, with which it is intimately connected. Huebert and Gorham (1983) found that Ca in the water column was essential for the survival of the macrophyte Potamogeton pectinatus, but only in the presence of other divalent cations, suggesting a protective role for external Ca.

Gipps and Coller (1980, 1982), using Chlorella pyrenoidosa Chick, found that Ca reduced both the short- (hours) and long- (days) term uptake of Cd. They concluded that Ca and Cd probably compete for the same uptake sites since they are both divalent metals with similar ionic radii. Khan et al. (1984) have shown that Ca and Cd have almost the same pattern of deposition in the cell walls, again suggesting a competitive interaction. Heuillet et al. (1988) reached similar conclusions using the alga Dunaliella bioculata, though an examination of the contour plots indicates that the effect of Ca was not pronounced. In terrestrial plants, Ca has been shown to antagonize Cd effects such as senescence and membrane deterioration in Avena sativa L. leaf segments (Fuhrer, 1983) and root uptake in Phaseolus vulgaris L. (Hardiman and Jacoby, 1984). Increasing the external Ca concentration also reduced Pb uptake in Hordeum vulgare L. and Festuca ovina L. (Garland and Wilkins, 1981) and promoted root growth in Zea mays L. in the presence of 4.2 μM Ni (Robertson, 1985).

This chapter examines Cd uptake, Cd toxicity and Cd by Ca interactions using a semi-continuous, flow-through culture system in axenic cultures of the submerged aquatic macrophyte Lemna trisulca.

MATERIALS AND METHODS

Axenic stock cultures of Lemna trisulca were maintained in 750 ml of filter-sterilized medium (Table 4.1) contained within 1-L Erlenmeyer flasks. Stock material was grown exponentially for 5 to 7 days before being used in experiments. For experiments,

three-frond fragments of L. trisulca were placed into 1-L Erlenmeyer flasks containing 750 ml of medium which had been filter-sterilized through 0.2 μ prewashed cellulose nitrate filters. The cultures were aerated at approximately 125 ml/min with room air passed through a humidifier containing deionized water, a cotton filter and a 0.45 μ sterile filter. Side-lighting was supplied continuously at 400 $\mu mol/m^2/s$ PAR by Sylvania cool-white fluorescent fixtures. The temperature was 25 ± 1°C in the growth chamber and 28 ± 2°C in the experimental flasks. Replacement of media occurred once or twice daily, increasing exponentially at a rate of 6 ml/frond/doubling time based on a 2 day doubling time. The replacement schedule per flask was as follows: 50 ml on day 4, 75 ml on day 6, 125 ml on day 7, 175 ml on day 8, 250 ml on day 9, 350 ml on day 10, 450 ml on day 11, 625 ml on day 12, 850 ml on day 13. The total volume of medium passing through a culture flask over the course of the 14 day experiment was 4 L. The initial pH was 7.8 ± 0.1 and, since no buffers could be used because of toxic effects (Chapter 2), the medium was designed to increase in pH to approximately 8.5 ± 0.3 by the end of the experiment. This range of pH changed the concentration of Cd^{+2} in the medium less than 5%, as calculated by MACS80 (Wagemann et al., 1990).

Plants were grown for 14 days at the specified Cd and Ca levels. Other nutrients were as described in Table 4.1. The phosphate was set at a level sufficient to support maximal growth (Chapter 3). The external Ca concentrations were set at 27.5, 55, 110, 220, or 440 μM, levels found at the lower end of the Ca range of natural waters. The lowest and highest Ca concentrations which were used encompassed the range of maximal growth of L. trisulca with respect to Ca under the specific conditions of this study (Chapter 3), even though they were low compared with levels found to support L. trisulca in natural waters (Landolt, 1986). The corresponding internal Ca concentrations, however, were 0.77, 1.04, 1.32, 1.67 and 2.21% dry wt. and were in the range of values reported for submerged aquatic plants by Hutchinson (1975), suggesting that the plants received adequate and normal amounts of Ca under these conditions.

Table 4.1. Standard nutrient medium used for stock cultures and experimental cultures. Stock solutions were made so that 1 ml/L produced the desired final concentration, except for Mg which was supplied at 2ml/L. Micronutrients were combined into one stock solution, as were EDTA and Fe. The Fe/EDTA stock solution was made by dissolving EDTA in distilled deionized water and then adding a slight excess of $FeCl_3$. The solution was then filtered to remove excess precipitated Fe. All stock solutions were kept in the dark at 4°C.

Nutrients	mg/L	μM	Micronutrients	mg/L	μM
KNO_3	25.2	250	$ZnSO_4 \cdot 7H_2O$	0.022	0.08
$CaCl_2 \cdot 2H_2O$	16.2	110	H_3BO_3	0.550	8.87
$MgSO_4 \cdot 7H_2O$	50.0	203	$CuSO_4 \cdot 5H_2O$	0.010	0.04
K_2HPO_4	2.61	15	$CoCl_2 \cdot 6H_2O$	0.010	0.04
$FeCl_3 \cdot 6H_2O$	2.43	9.0	$Na_2MoO_4 \cdot 2H_2O$	0.170	0.70
$Na_2EDTA \cdot 2H_2O$[a]	3.35	9.0	$MnCl_2 \cdot 4H_2O$	0.180	0.91
$NaHCO_3$	42.0	500			

a. Disodium ethylenediaminetetraacetic acid

Cadmium was set at nominal concentrations of 0, 0.04, 0.08, 0.16, 0.32, 0.64, or 1.28 µM. The Cd concentrations were chosen to range from levels producing no significant effect to a level producing a 95% decrease in final yield. Each experiment, therefore, consisted of a 5x7 factorial design of varying Cd or Ca concentrations with three replicates per treatment. The measured Cd concentrations at the end of the experiment are listed in Table 4.2 for the 35 different treatments. The accuracy was 95% with a range of 83 to 111% and the precision (as coefficient of variation) was 11% with a range of 1.2 to 24.5%. The nominal Cd concentrations were therefore achieved and maintained over the 14 day culture period. MACS80 indicated that greater than 99% of Cd was bound as Cd:EDTA if excess EDTA was available. In the absence of available EDTA, approximately 98% of the Cd was present as Cd^{+2} under the conditions of this study. The culture medium was therefore carefully designed with a 1:1 ratio between iron and EDTA in an attempt to reduce the binding of all other cations to EDTA, since the formation constant for Fe:EDTA is approximately 25.1, many orders of magnitude greater than the formation constant of any other cation in the medium (Skoog and West, 1969). Unfortunately, the exact speciation of cations in the medium over time could not be determined since both Fe:EDTA and EDTA are not stable in light at alkaline pH values (Lockhart and Blakely, 1975; Frank and Rau, 1990).

Plants were removed from the culture flasks after 14 days and the fronds counted. A frond was tallied if the root bud was visible below the blade of the frond. The multiplication rate ($MR=1000*[\log F_t - \log F_o]/t$) was measured on frond production as defined by Hillman (1961) where F_t equals the final frond number, F_o equals the initial frond number, and t equals the culture time in days.

In a preliminary experiment, plants were exposed to 0.32 µM Cd and washed three times for one minute with either distilled H_2O, 1 mM EDTA, 1 mM sodium dodecyl sulphate (SDS), or 1 mM HCl to determine the effect of different washing procedures on the Cd content of L. trisulca. No attempt was made to remove free-space cations as in

Table 4.2. Cadmium concentration (nM) in flask sampled on day 13. Mean ± sd, n = 3.

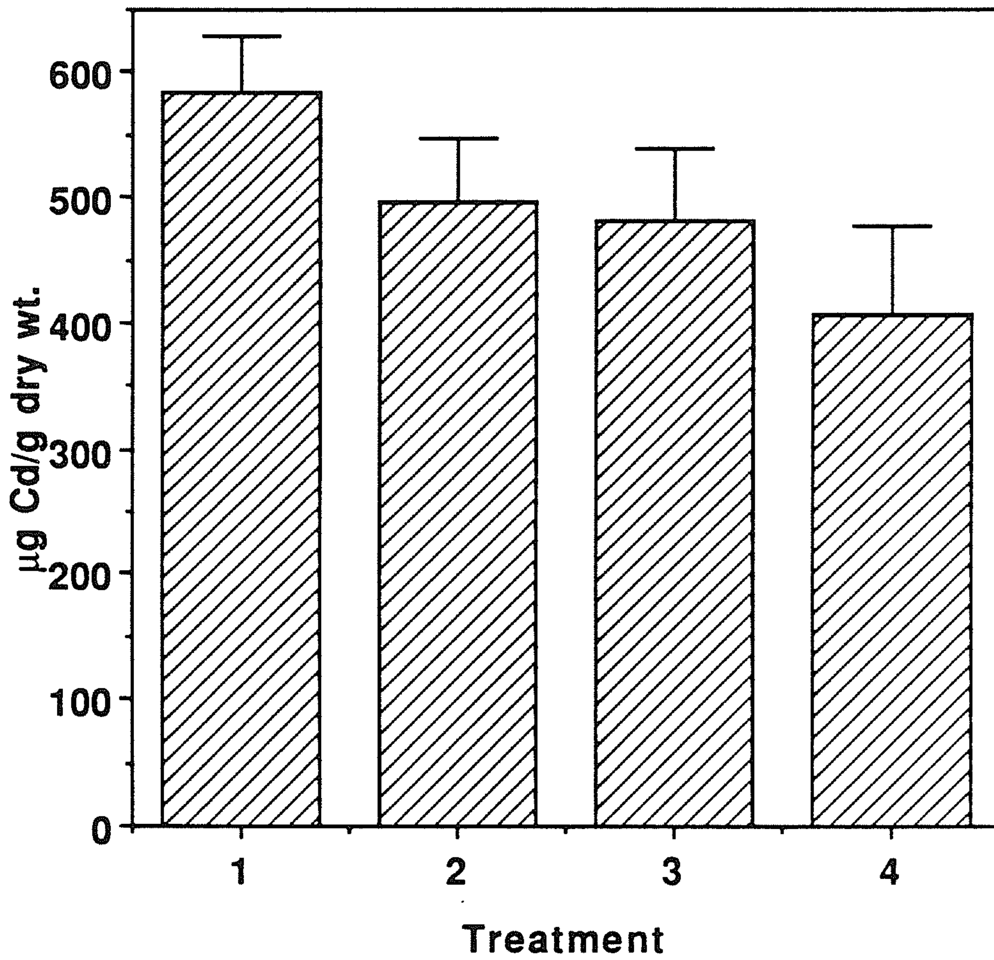
Cadmium	Calcium (µM)				
(nM)	27.5	55	110	220	440
0	0.0	0.0	0.1	0.0	0.0
40	44 ± 5	38 ± 6	34 ± 8	36 ± 4	36 ± 4
80	83 ± 8	75 ± 5	89 ± 13	83 ± 1	67 ± 4
160	170 ± 7	160 ± 21	140 ± 2	150 ± 3	140 ± 4
320	310 ± 19	290 ± 71	290 ± 20	300 ± 26	300 ± 5
640	600 ± 69	580 ± 77	580 ± 140	530 ± 36	630 ± 68
1280	1300 ± 98	1300 ± 160	1200 ± 120	1200 ± 130	1200 ± 110

Kwan and Smith (1991) where L. minor plants were washed for 20 min with ice-cold $LaCl_3$. The difference between these four washing treatments was marginally significant using one-way ANOVA ($p = 0.033$, 3df, $F = 4.84$). The plants washed with SDS had the lowest Cd content of 406 ± 71 µg Cd/g dry wt. and the plants washed with distilled water contained the highest Cd at 584 ± 45 µg Cd/g dry wt. (Fig. 4.1). Both of these values, however, are in the range of Cd contents found throughout this study (Fig. 4.6, 5.8, 7.5,Table 6.2).

For experiments, plants were washed three times in distilled water and dried for 24 hours at 85°C in 40 ml ceramic crucibles to determine oven dry weight. Concentrated HNO_3 was added and heated to dryness to prevent the volatilization of Cd by the formation of $CdNO_3$, after which the crucibles were heated at 450°C for 6 h. The ash samples were dissolved in heated 5N HCl, filtered with Whatman #42 ash-free filter papers and diluted to 100 ml with deionized water. Calcium was determined by atomic absorption spectroscopy and Cd by a Varian GTA-95 graphite furnace using background correction and an injection volume of 8 µl. The precision of the Cd measurement, based on 24 duplicates over the range of absorbance, was 4.4 ± 5.7% (as coefficient of variation). On spiked plant samples, the recovery of Cd was measured at 114 ± 7%, with a coefficient of variation of 5.9%. All reagents were ACS grade or better. Glassware was acid washed in 3N HNO_3 and rinsed repeatedly with deionized water. The water used throughout was distilled and deionized with a final conductivity of approximately 0.06 µS/cm.

The data for final yield, frond weight and plant Cd content were log transformed to stabilize the variance and, together with the data for MR, were tested by ANOVA for significance of treatment effects. In addition, data from the replicates were analyzed for differences in response. The Cd EC50 values for MR and final yield were estimated by linear regression. A trends test (Tukey et al., 1985) was used to calculate the threshold level of toxicity for both internal and external Cd levels. For internal Cd the doses, or

Figure 4.1. The cadmium content of Lemna trisulca plants exposed to 0.32 μM cadmium and washed by four treatments: 1; distilled H_2O, 2; 1 mM HCl, 3; 1mM EDTA, 4; 1mM SDS.



µg Cd/g dry wt.
600
500
400
300
200
100
0
1
2
3
4
Treatment

categories, were arbitrarily chosen as 21 groups of 5 observations each. Tukey et al. (1985) defines the no-statistical-significance-of-trend dose (NOSTASOT) as the highest dose at which no statistically significant trend is observed. The NOSTASOT corresponds to the no-observable-effect-concentration (NOEC) as defined by Rand and Petrocelli (1985). Data from this chapter are contained in Table A.3 in Appendix A. This chapter has been published as Huebert and Shay (1991b).

RESULTS

The MR (Table 4.3) and final yield (Fig. 4.2) in mg dry wt. of L. trisulca plotted against the external Ca and Cd concentration showed little effect of external Ca on Cd toxicity. The MR in the five control treatments (without Cd) averaged 149 ± 5 (Table 4.3). Coefficient of variation values ranged from 1.4 to 14% in the 35 treatments. The MR decreased to a low of approximately 60 at the highest Cd concentration. The final yield ranged from 88 to 98 mg dry wt. after 14 days growth. The yield dropped to approximately 5 mg dry wt. at the highest Cd concentration. This was a decrease of 95% in biomass production. An ANOVA (Table 4.4) indicated that there was no significant ($p \geq 0.05$) effect of Ca, or Cd by Ca interaction, on the MR or final yield in L. trisulca.

The estimated EC50 for the MR of L. trisulca under the specific conditions of this study was 0.99 µM external Cd (Fig. 4.3a). The EC50 for the final yield over the 14 days growth period was 0.56 µM external Cd (Fig. 4.3b). The relationship between the final yield EC50 and the percentage reduction in MR (Fig. 4.4) shows that the longer the culture time and the faster the MR of control treatments, the lower the Cd concentration needed to produce a final yield EC50. The EC50 for final yield at 14 days occurred at a Cd concentration producing a 15% reduction in MR when the control doubled every 2 days (Fig. 4.4). If the plant had been cultured for only seven days, the final yield EC50 would have occurred at a Cd concentration which reduced the MR 28%.

Table 4. 3. Multiplication rate of Lemna trisulca in response to external Ca and Cd concentration. Mean ± sd, n = 3.

Cadmium	Calcium (μM)				
(μM)	27.5	55	110	220	440
0	152 ± 4	152 ± 3	150 ± 3	146 ± 4	144 ± 2
0.04	145 ± 8	147 ± 2	148 ± 6	142 ± 6	139 ± 8
0.08	146 ± 4	150 ± 4	148 ± 6	138 ± 3	132 ± 2
0.16	140 ± 10	140 ± 6	136 ± 11	141 ± 8	134 ± 2
0.32	111 ± 15	111 ± 12	122 ± 3	117 ± 6	119 ± 9
0.64	70 ± 5	71 ± 7	82 ± 10	82 ± 7	76 ± 7
1.28	57 ± 3	59 ± 2	62 ± 2	57 ± 2	66 ± 2

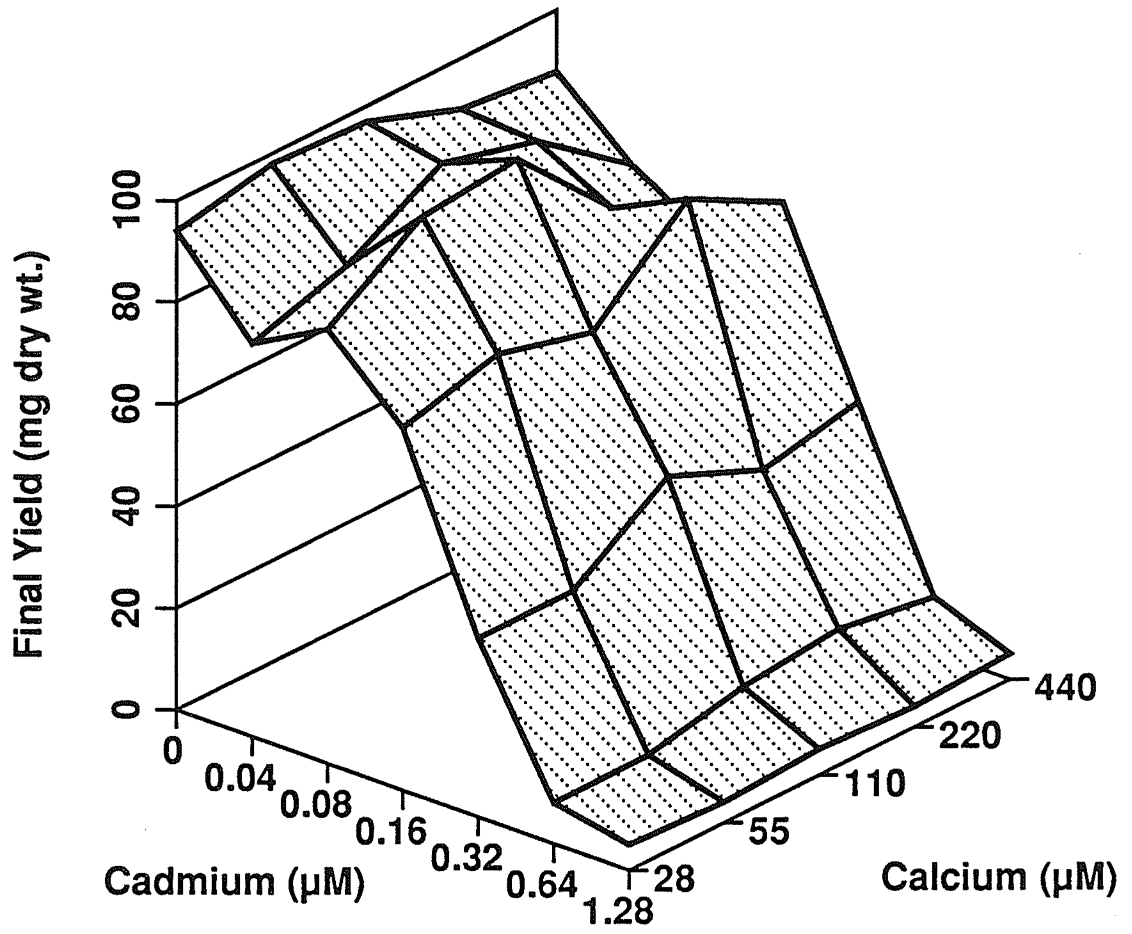
Table 4. 4. Analysis of variance on the response of *Lemna trisulca* to Cd and Ca for MR, final yield (as log mg dry wt.), Cd content (as log μg Cd/g dry wt.) and frond weight (as log μg/frond).

Source	df	MS	F	P
MR				
cadmium	6	19430	417	0
calcium	4	89	1.91	0.119
Cd vs Ca	24	69	1.48	0.105
Error	70	47		
Final yield				
cadmium	6	4.246	350	0
calcium	4	0.017	1.42	0.237
Cd vs Ca	24	0.010	0.82	0.706
Error	70	0.012		
Cd content				
cadmium	6	16.2	480	0
calcium	4	0.070	2.06	0.111
Cd vs Ca	24	0.028	0.80	0.726
Error	68	0.034		
Frond weight				
cadmium	6	0.049	12.8	0
calcium	4	0.009	2.28	0.069
Cd vs Ca	24	0.004	1.05	0.420
Error	70	0.004		

Table 4.5. Probability values for the determination of the Cd NOEC in *Lemna trisulca* for MR and final yield.

	Cadmium (µM)				
	1.28	0.64	0.32	0.16	0.08
MR	0	0	0	0.001	0.038
Final yield	0	0	0	0.002	0.121

Figure 4.2. The effect of calcium and cadmium on the final yield (mg dry wt.) of *L. trisulca*.



Final Yield (mg dry wt.)
100
80
60
40
20
0
0
0.04
0.08
0.16
0.32
0.64
1.28
Cadmium (µM)
28
55
110
220
440
Calcium (µM)

Similarly, if the plant had doubled every 3 days, the final yield EC50 would have occurred at a Cd concentration which reduced the MR 23%.

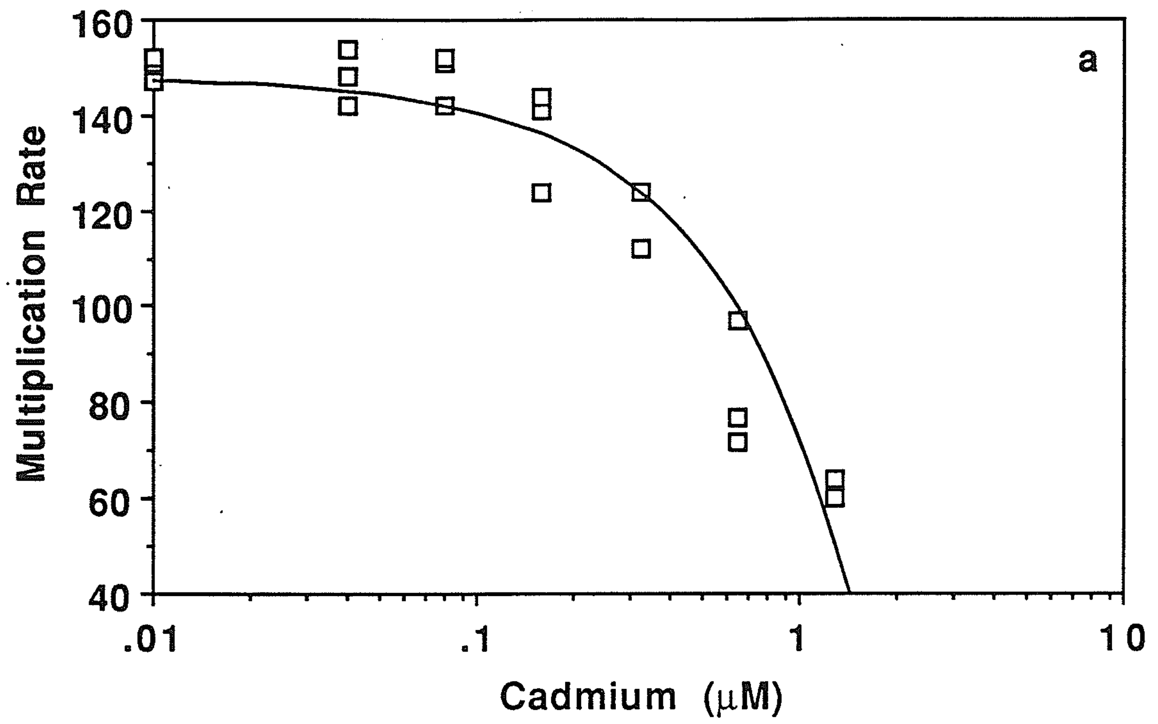
A trends test (Tukey et al., 1985) on data combined over the range of the five Ca concentrations indicated that the NOEC was 0.08 μM ($p \leq 0.01$) external Cd for both MR and final yield (Table 4.5). The lowest-observable-effect-concentration (LOEC) would then be the next highest tested Cd concentration, or 0.16 μM Cd. The trends test, unlike the EC50, gave the same result for both MR and final yield.

A gradual rise in the average weight of fronds occurred with increasing Ca and Cd concentration, from 233 μg/frond at the lowest Ca and Cd levels up to approximately 310 μg/frond at 0.32 μM Cd (Fig. 4.5). At higher Cd concentrations the frond weight began to decrease to a low of approximately 220 μg/frond. The weight of individual fronds of L. trisulca responded significantly ($p \leq 0.05$) to the external Cd concentration (Fig. 4.5, Table 4.4) and slightly but not significantly to the external Ca concentration ($p = 0.069$). There was, however, no significant ($p \geq 0.05$) interaction between external Cd and Ca.

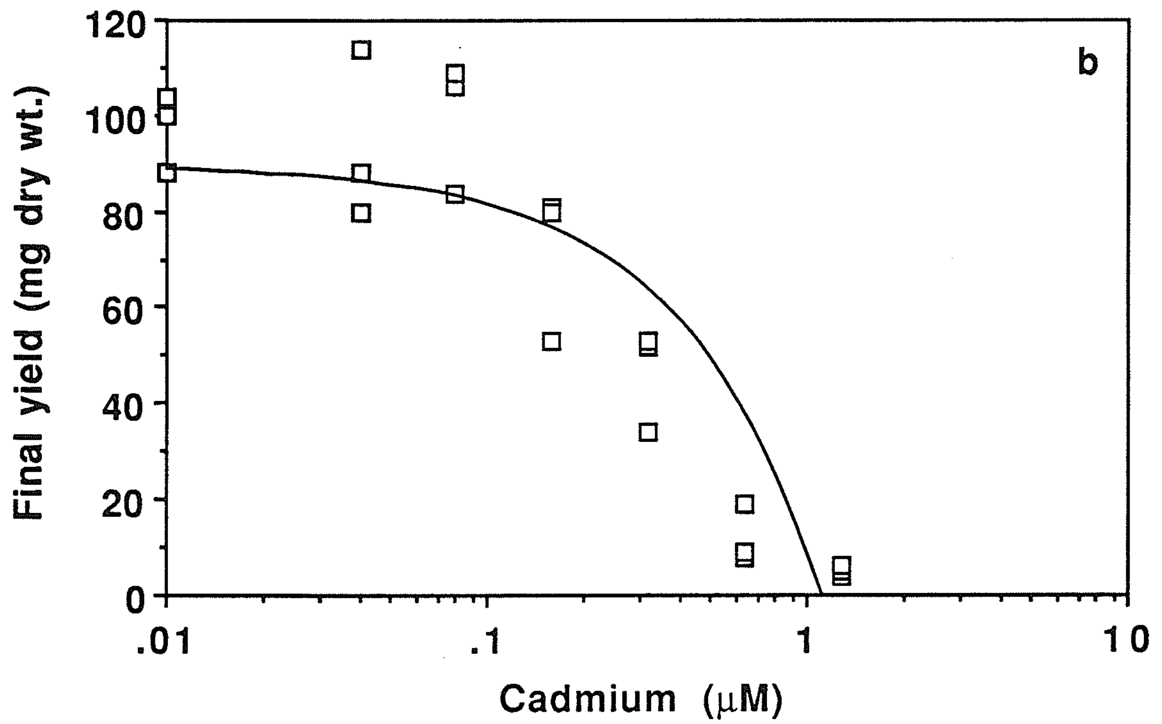
The internal Cd content plotted against the external Ca and Cd concentration clearly showed little effect of Ca on Cd uptake (Fig. 4.6). An ANOVA (Table 4.4) indicated no significant ($p \geq 0.05$) effect of Ca or interaction between external Cd and Ca on the internal Cd content. The content of Cd in L. trisulca saturated at between 1,500 to 3,000 μg Cd/g dry wt. which occurred between 0.64 and 1.28 μM external Cd. At 0.04 μM external Cd, the Cd content ranged from 30 to 80 μg Cd/g dry wt.

The largest amount of Cd removed by L. trisulca was 21.6 μg from cultures containing 0.32 μM external Cd (Table 4.6). Uptake above 0.32 μM Cd was reduced due to the sharp drop in the biomass production of L. trisulca. The highest average concentration factor (μg Cd/g dry wt.: nominal Cd concentration in the medium) was 24,800 which occurred at 0.64 μM external Cd (Table 4.6). Concentration factors could be expressed on a wet weight basis by dividing by 14.1. The percentage of total available Cd removed from the nutrient medium peaked at 22.8% at the lowest external Cd level of

Figure 4.3. The effect of external cadmium on multiplication rate (MR) (fig.4.3.a) and final yield (Fig. 4.3.b) for Lemna trisulca. For MR, y = 148 - 75x, R^2 = 0.90. For final yield, y = 89 - 79x, R^2 = 0.77.



Multiplication Rate
160
140
120
100
80
60
40
a
.01
.1
1
10
Cadmium (μM)



Final yield (mg dry wt.)
120
100
80
60
40
20
0
b
.01
.1
1
10
Cadmium (μM)

Figure 4.4. Relationship between the percent reduction in multiplication rate (MR) caused by a toxicant and the number of days of exponential growth required to produce a final yield EC50. Three different control MR are represented. For a one day doubling time (MR = 300), y = 2.0 - 1.0x; for a two day doubling time (MR = 150), y = 2.3 - 1.0x; for a three day doubling time (MR = 100), y = 2.5 - 1.0x. The faster the control MR and the longer the culture time, the lower the percent reduction in MR and the lower the concentration of a toxicant needed to produce a final yield EC50.

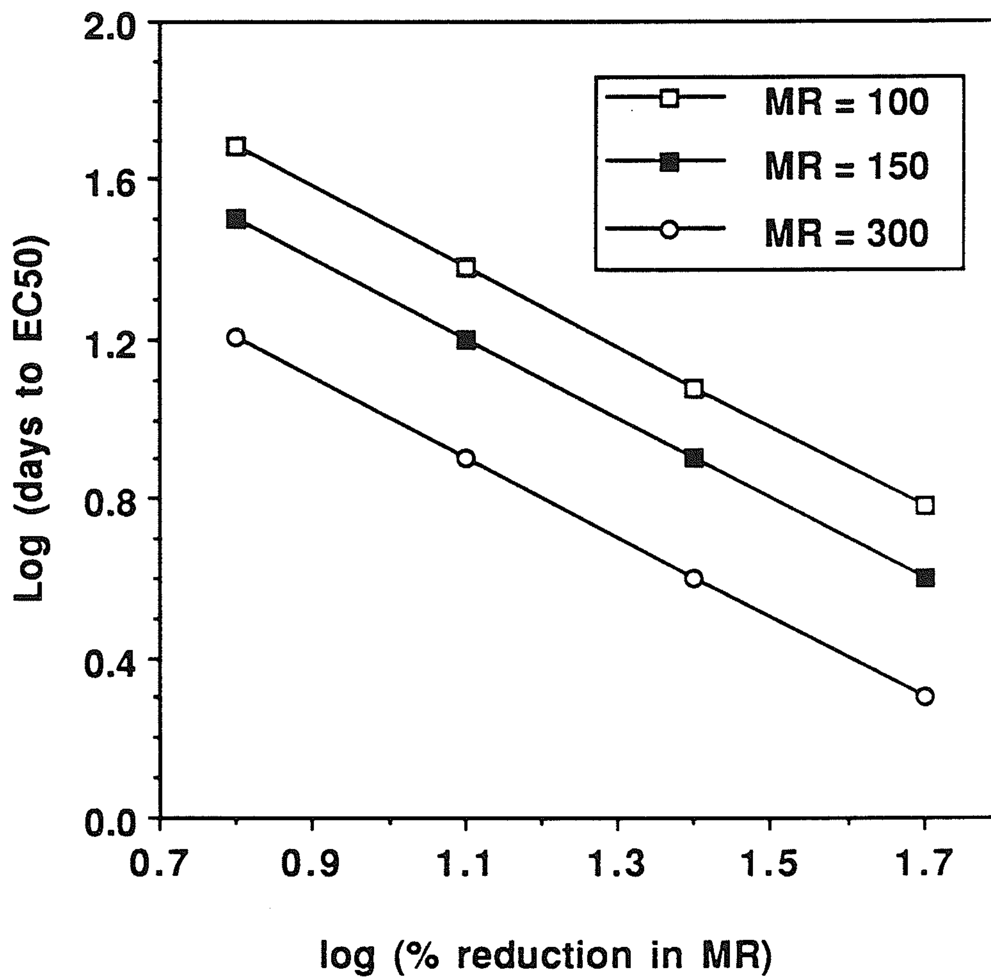
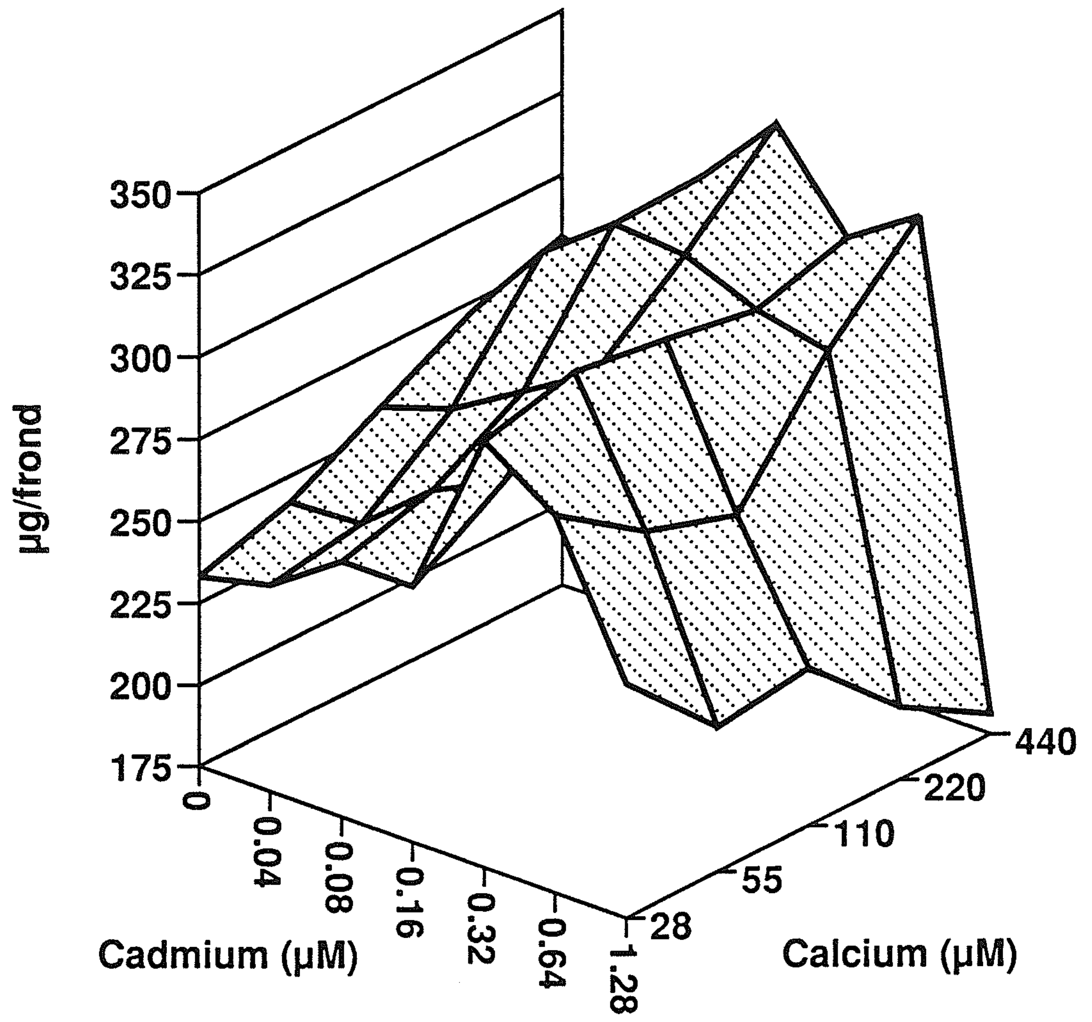
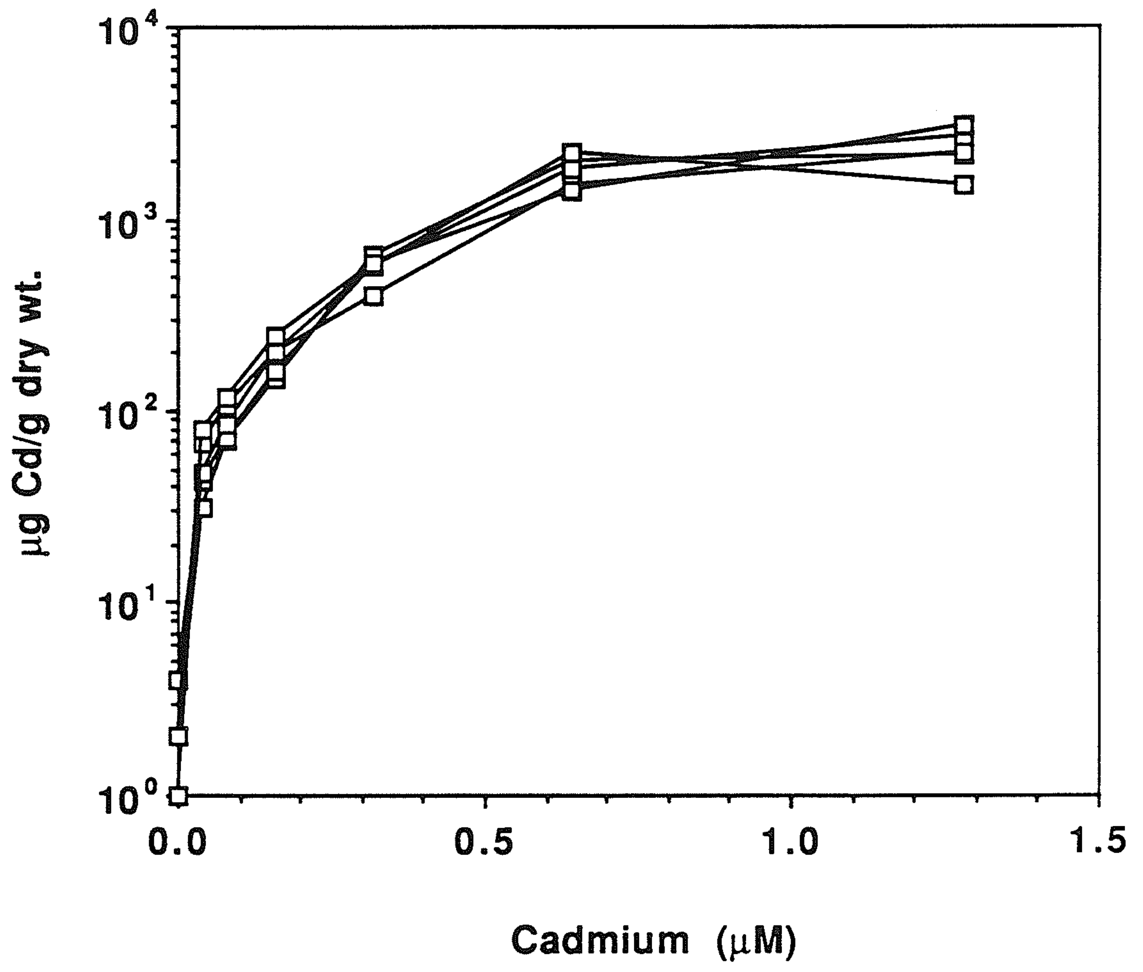

2.0
1.6
1.2
0.8
0.4
0.0
0.7
0.9
1.1
1.3
1.5
1.7
Log (days to EC50)
log (% reduction in MR)
MR = 100
MR = 150
MR = 300

Figure 4.5. The effect of calcium and cadmium on the frond weight (μg/frond) in Lemna trisulca.



350
325
300
275
250
225
200
175
µg/frond
0
0.04
0.08
0.16
0.32
0.64
1.28
Cadmium (µM)
440
220
110
55
28
Calcium (µM)

Figure 4.6. The effect of calcium and cadmium on cadmium uptake (μg Cd/g dry wt.) in Lemna trisulca at the five calcium concentrations. The lines are almost identical at each calcium concentration and have not been separately identified.



μg Cd/g dry wt.
10^4
10^3
10^2
10^1
10^0
0.0
0.5
1.0
1.5
Cadmium (μM)

0.04 μM and declined steadily with increasing external Cd to a low of 1.7% at 1.28 μM Cd (Table 4.6).

The MR remained constant at 147 ± 6 as the internal Cd content increased from near the detection limit to 116 ± 4 μg Cd/ g dry wt. (Fig. 4.7). Internal Cd contents above this value were associated with a significant ($p \leq 0.01$) decrease in the MR (Table 4.6). The NOEC-LOEC was therefore in the ranges with means of 116 to 147 μg Cd/ g dry wt.

DISCUSSION

Significant reductions in MR and final yield in L. trisulca first occurred at an external Cd concentration of 0.16 μM. This compares with the concentration of 0.09 μM at which a reduction in growth was initially observed in Salvinia natans (Hutchinson and Czyrska, 1975). However, 0.16 μM is lower than the values for initial toxicity reportedby other researchers, which range from 0.45 μM in L. valdiviana and L. minor (Hutchinson and Czyrska, 1975; Wang, 1986a) to over 9.0 μM in L. gibba and Spirodela polyrhiza (Polar and Kucukcezzar, 1986; Srivastava and Jaiswal, 1990).

The reasons for the wide range in concentrations at which toxic effects are first measured are probably due in part to species differences and the processes tested but may perhaps also be ascribed to culture conditions (see review in Landolt and Kandeler, 1987). My study took place under axenic conditions with a high initial volume:plant ratio of 750 ml of medium to an inoculum of approximately 750 μg (three fronds). In addition, the regular replacement of media ensured that the Cd concentrations were maintained (Table 4.3) and nutrient levels (Chapter 2 and 3) were optimal and remained relatively constant over the 14 day growth period. Studies using batch culture and particularly those carried out under non-axenic conditions may be confounded by changes in Cd content, nutrient conditions and dissolved organic carbon over the time of the experiment. Large differences between replicates are to be expected due to changes in Cd speciation and

Table 4.6. Characteristics of Cd uptake in Lemna trisulca under the specific conditions of this study. Mean ± sd, n = 15.

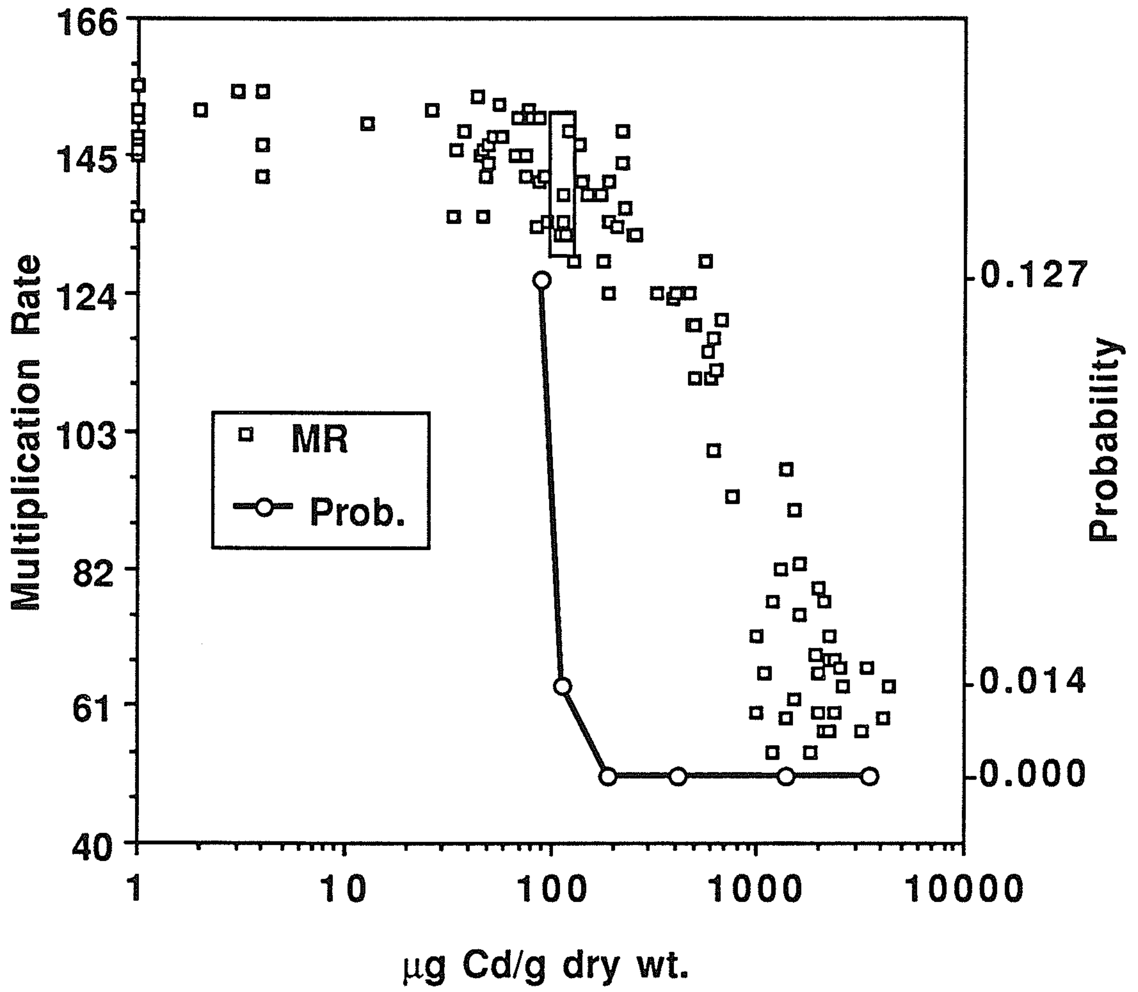
Cadmium (μM)	% removed[a]	Concentration Factor ($x10^3$)[b]	Total μg Cd[c]
0.04	22.8 ± 7.0	11.2 ± 4.2	4.1 ± 1.3
0.08	21.0 ± 3.4	10.1 ± 2.4	7.5 ± 1.2
0.16	20.5 ± 5.9	10.7 ± 2.3	14.7 ± 4.2
0.32	15.0 ± 3.4	15.5 ± 3.5	21.6 ± 4.9
0.64	6.3 ± 2.2	24.8 ± 8.6	18.0 ± 6.3
1.28	1.7 ± 0.7	16.0 ± 6.7	10.1 ± 4.1

a. The amount of Cd removed from the culture medium by L. trisulca expressed as a percentage of the total available Cd in the 4 L of media.

b. Values can be converted to fresh weight,concentration factors by dividing by 14.1.

c. The total amount of Cd (μg) removed by L. trisulca after 14 days growth at the specified external Cd concentrations.

Figure 4.7. The effect of internal cadmium content (μg Cd/g dry wt.) on multiplication rate in Lemna trisulca. Probability levels calculated for the NOEC are represented by the solid line. The rectangle encloses the last category for which there was no significant trend in the data.



166
145
124
103
82
61
40
Multiplication Rate
0.127
0.014
0.000
Probability
MR
Prob.
1
10
100
1000
10000
µg Cd/g dry wt.

excreted algal metabolites (Gipps and Coller, 1980). Hall (1986), testing the toxicity of copper in two species of chlorophytes, found an increase in sensitivity of four orders of magnitude going from batch to chemostat cultures. It is essential, therefore, that as great a control as possible be exercised over the culture conditions in order to minimize uncontrolled changes in metal concentrations and speciation, especially when definitive values of toxicity are being calculated (as in Wang, 1986a).

The EC50 under the specific conditions of this study was 0.56 μM Cd for final yield and 0.99 μM Cd for MR. The discrepancy between EC50 values based on MR and final yield exists because percent reduction in final yield depends on several factors. These include the reduction in growth caused by a toxicant, the length of time the plants are cultured and on MR of the control treatment. The faster the control MR and the longer the culture time, the lower the concentration of toxicant needed to produce a final yield EC50.

This relationship is relevant to the environment because it means that even a reduction in MR that is barely detectable will result in a large reduction in standing crop after only a few weeks of growth. The NOEC is therefore the most important toxicant concentration from a regulatory perspective, since it is the highest toxicant concentration that will not decrease standing crop.

Percent reduction in final yield is the parameter used for regulatory purposes to assess the effect of toxicants on duckweeds (U. S. EPA, 1985; Wang, 1986a; Hughes et al., 1988; Taraldsen and Norberg-King, 1990; Wang, 1990; ASTM, 1991; Wang, 1991). This is inappropriate for the reasons discussed above. This reduction is particularly applicable in these seven studies since culture times vary from four (Wang, 1986a; Taraldsen and Norberg-King, 1990; Wang, 1990) to seven days (U. S. EPA, 1985; ASTM, 1991), and the basal growth rates of aquatic macrophytes are not constant over time even under seemingly identical conditions (Huebert and Gorham, 1983; Landolt and Kandeler, 1987; Wang, 1990; Chapter 6). For instance, Wang (1990) found that the

doubling time of L. minor varied from 1.3 to 2.8 days over an eighteen month period and I have found (Chapter 6) that the doubling time of L. trisulca varied from 1.6 to 2.4 days despite rigorously controlled conditions. Studies which use percent reduction in final yield to assess toxicity are therefore confounded by factors extraneous to the actual effect of the toxicant.

The average weight of individual fronds of L. trisulca responds to the external nutrients (Chapter 3). In this study, the frond weight also responded significantly to the external Cd concentration. This is contrary to the results of Nasu et al. (1984) who found that frond weight responded to Cu but not to the Cd concentration.

Lemna trisulca accumulated approximately 30 to 80 μg Cd/g dry wt. at the lowest Cd concentrations tested and 3,000 μg Cd/g dry wt. at the highest external Cd concentrations. This latter value is higher than that of L. valdiviana but lower than S. natans (Hutchinson and Czyrska, 1975). Van der Werff and Pruyt (1982), using four species, reported 10 to 120 μg Cd /g dry wt. at 0.10 μM Cd and up to 1,100 μg Cd/g dry wt. at 1.0 μM Cd.

Lemna trisulca concentrated Cd in its tissues up to 24,800 times that of the growth medium at 0.64 μM Cd. This is contrary to the generally held opinion (see review in Landolt and Kandeler, 1987) that concentration factors increase as metal concentrations increase. Because of increased biomass production at lower Cd concentrations, however, the cultured plants removed the greatest amount of Cd at only 0.32 μM and were most efficient at removing Cd from the medium at the lowest Cd concentration tested. Landolt and Kandeler (1987) have compiled a list of concentration factors for a variety of metals and different species of Lemnaceae. For Cd, values ranged from 170 to 25,000. The results of my work are at the high end of this range.

In my study, a NOEC of 116 μg Cd/g dry wt. was calculated on the internal Cd content. It is possible that such a calculated value could be used to assess the potentially toxic effect of metals in natural systems. This approach must be used with caution,

however, since the phosphate nutrient status may affect the internal toxicity of metal ions (Hall, 1986; Lazinsky and Sicko-Goad, 1990; see also Chapter 6).

In addition, more information is needed on the accuracy of the metal content determination. This concern would be particularly important if one wanted to use metal contents in a predictive way since the method of washing the plants prior to analysis may alter the metal content. In my study, however, washing with SDS, EDTA, HCl or distilled H_2O had little effect on the measured Cd content of the plant. A more rigorous washing procedure such as the one described by Kwan and Smith (1991) to remove free-space ions may have produced a different result.

This study found that there were no significant effects of Ca or interactions between Ca and Cd with respect to the MR, final yield, uptake of Cd or weight of fronds of L. trisulca under the conditions of this study. These results are contrary to previous studies, all of which showed that Ca mitigated metal toxicity in several different species for a wide variety of processes. For algae, it has been suggested that the mitigation of Cd toxicity by Ca occurs because both are divalent cations with similar ionic radii (Gipps and Coller, 1982) and would therefore compete for the same binding sites. My work suggests that, at least for L. trisulca, Cd and Ca do not interact or compete for binding sites. This further suggests that the mechanism of Cd toxicity involves processes removed from the action of Ca, perhaps involving other cations such as Zn or other micronutrients. This problem will remain unresolved until further experiments are undertaken on interactions between Cd and other nutrients, and on the site of Cd toxicity and its localization within L. trisulca.

Chapter 5. Zinc toxicity and its interaction with cadmium

INTRODUCTION

Zinc is used in a variety of industrial processes. It enters aquatic environments in mining, industrial and domestic effluents (Cammarota, 1980). Zinc reduces the growth of aquatic plants at concentrations ranging from approximately 1.5 μM in some marine diatoms (Braek et al., 1980) to approximately 150 μM in several species of floating aquatic macrophytes (Nasu and Kugimoto, 1981; Schreinemakers, 1984; Sela et al., 1989; Jain and Jha, 1990).

Zinc and Cd are IIB metals that occur naturally together in the same ore types; Cd is refined from Zn ores (Nriagu, 1980). It is of interest to examine the toxicity of mixtures of Zn and Cd because of their similarities and because Zn is an essential element for plant growth while Cd is not. Synergistic effects between Zn and Cd have been found in the alga Hormidium rivulare Kütz (Say and Whitton, 1977), for several marine diatoms (Braek et al., 1980) and in the aquatic plants Lemna minor and Salvinia natans (Hutchinon and Czyrska, 1975). Braek et al. (1980) also found antagonistic and additive interactions between Zn and Cd, depending on the plant species. In perhaps one of the most sophisticated studies examining the interaction between Zn and Cd, Bennett and Brooks (1989) employed a turbidostat culture technique using the alga Chlorella pyrenoidosa and clearly showed that Zn antagonized Cd toxicity at levels of Zn which were themselves not toxic.

The purpose of this chapter was to examine Zn uptake and toxicity and to determine if there was a Zn by Cd interaction in the submerged aquatic macrophyte Lemna trisulca grown in axenic cultures in which a portion of the medium was replaced daily.

MATERIALS AND METHODS

Axenic stock cultures of Lemna trisulca were maintained in 750 ml of filter-sterilized medium and grown exponentially for 5 to 7 days before being used in experiments. The growth medium contained 250 µM KNO_3, 110 µM $CaCl_2$, 203 µM $MgSO_4$, 15 µM K_2HPO_4, 500 µM $NaHCO_3$, 8.87 µM H_3BO_3, 0.91 µM $MnCl_2$, 0.70 µM Na_2MoO_4, 0.08 µM $ZnSO_4$, 0.04 µM $CuSO_4$ and 0.04 µM $CoCl_2$. Experiment 1 examined Zn toxicity and Experiment 2 examined the interaction between Zn and Cd. For experiments, a L. trisulca plantlet consisting of a single frond and root, with a dry weight of approximately 250 µg, was placed into either a 250-ml Erlenmeyer flask containing 150 ml of filter-sterilized medium (Exp. 1) or a 1-L Erlenmeyer flask containing 750 ml of filter-sterilized medium (Exp. 2). The cultures were aerated at approximately 125 ml/min with ambient air passed through a humidifier containing deionized water, a cotton filter and a 0.45 µ sterile filter. Light was supplied from the side of the flask continuously at 400 µmol/m^2/s PAR by Sylvania HO cool-white fluorescent lamps. The temperature was 25 ± 1°C in the growth chamber and 28 ± 2°C in the experimental flasks. Replacement of the medium occurred once or twice daily, increasing exponentially at a rate based on a 2-d doubling time. The total volume of medium passing through the culture flasks over the course of the experiment was 2 L for Experiment 1 and 4 L for Experiment 2. The initial pH was 7.8 and, since no buffers were used because of toxic effects (Chapter 2), the pH rose gradually to approximately 8.3 ± 0.3 by the end of the experiment. This was probably because NH_4 was replaced by NO_3 in the medium.

Plants were grown for 14 days at the specified Zn (Exp. 1) or Zn and Cd levels (Exp. 2). In Experiment 1, the Zn concentration ranged from 0.08 to 24.5 µM. In Experiment 2, Zn was set at nominal concentrations of 0.08, 3.06, 6.12 and 12.2 µM and Cd was set at 0, 0.16 or 0.32 µM. Experiment 2, therefore, consisted of a 4 by 3 factorial design of varying Cd and Zn concentrations. Zinc and Cd concentrations were chosen in

order to examine Zn by Cd interactions at threshold levels of toxicity for Zn and Cd and at levels producing a 50% reduction in final yield (Zn and Cd) and growth rate (Zn). Experiment 2 was repeated three times and the replicates blocked for time in a randomized complete block design.

The Zn and Cd concentrations in the culture flasks were measured on day 14 (Table 5.1). The accuracy was 97% for Zn and 101% for Cd. The precision (as coefficient of variation) was 5.8% for Zn and 9.8% for Cd. This indicates that the nominal concentrations were achieved and maintained over the 14 day culture period. Plants were removed from the culture flasks after 14 days and fronds with a visible root were counted. Plants were then washed three times in distilled water and dried for 24 h at 85°C in 50 ml beakers to determine final yield in mg dry wt. Concentrated HNO_3 was added and heated to dryness four times, after which the material was dissolved in heated 5N HCl, filtered with Whatman #42 ash-free filter papers and diluted to 100 ml with deionized water. Zn was determined by atomic absorption spectroscopy and Cd with a Varian GTA-95 graphite furnace connected to a Varian AA-975 atomic absorption spectrophotometer. Background correction and an injection volume of 15 µL was used. All reagents were ACS grade or better. Glassware was acid washed in 3N HNO_3 and rinsed thoroughly with deionized water. The water used had a conductivity of approximately 0.06 µS/cm.

In Exp. 1, EC50 values were estimated by linear regression. In Experiment 2, the data for final yield and plant Cd and Zn content were log transformed to stabilize the variance and, together with the data for MR, tested by ANOVA for significance of treatment effects. In addition, data from the three complete blocks were analyzed for differences in response so that variation due to time could be removed from the analysis. The MR data were also examined using a second-order multiple regression model. One-way ANOVA was used to assess differences at single Zn concentrations across all three Cd levels to determine where Zn by Cd interactions occurred. The data for

Table 5.1. Zinc and Cd concentrations (μM) in culture flask at each of twelve treatments of varying Cd and Zn concentration in Exp. 2. Samples were taken on day 14. Mean ± sd, n = 3. For any treatment, the top value is for Cd and the bottom one is for Zn.

Cadmium	Zinc			
	0.08	3.06	6.12	12.2
0	0	0	0	0
	-----[a]	2.9 ± 0.2	5.6 ± 0.2	12.4 ± 0.6
0.16	0.16 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.18 ± 0.02
	-----	2.8 ± 0.2	5.8 ± 0.4	12.5 ± 0.8
0.32	0.30 ± 0.03	0.31 ± 0.02	0.31 ± 0.03	0.32 ± 0.03
	-----	2.9 ± 0.2	6.0 ± 0.3	12.5 ± 0.6

a. Analyses were not performed for control levels of Zn.

Experiment 2 are presented as the mean ± one s.d., n = 3. Data from this chapter are contained in Table A.4 in Appendix A. This chapter has been published as Huebert and Shay (1992a).

RESULTS

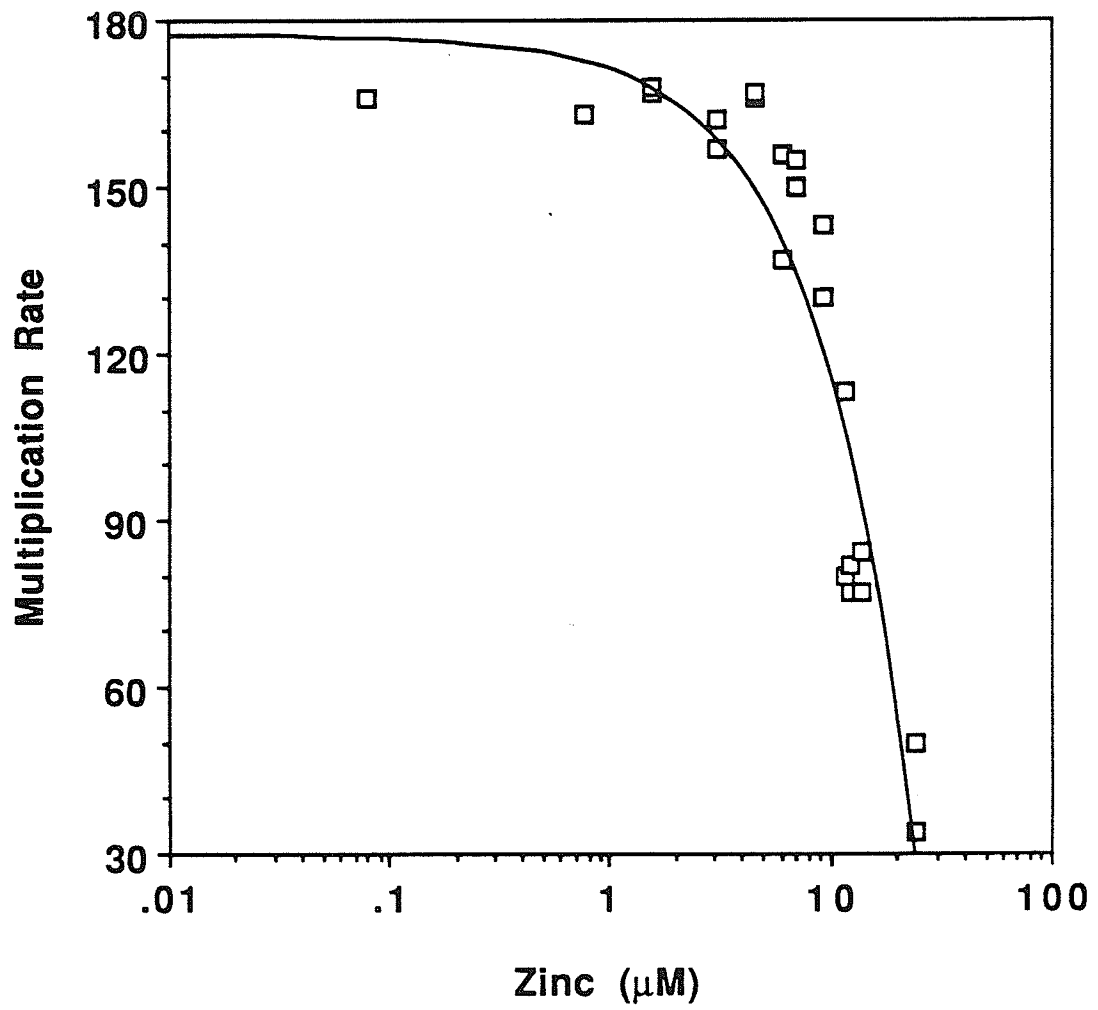
Zinc toxicity (Exp. 1)

Concentrations higher than about 4.5 μM Zn resulted in a reduced MR and final yield for L. trisulca (Fig. 5.1 and 5.2). Control treatments grown at 0.08 μM Zn had an MR of 166 ± 8 and a final yield of 56 ± 16 mg dry wt. The MR decreased steadily to a low of 34 at 24.5 μM Zn, a rate at which only three fronds weighing 1 mg dry wt. were produced after 14 days growth. Fronds were bleached at this latter concentration, though the meristems still appeared green and viable. The EC50 was estimated at 16 μM Zn for MR (Fig. 5.1) and at 7.8 μM Zn for final yield (Fig. 5.2).

Zinc by cadmium interactions (Exp. 2)

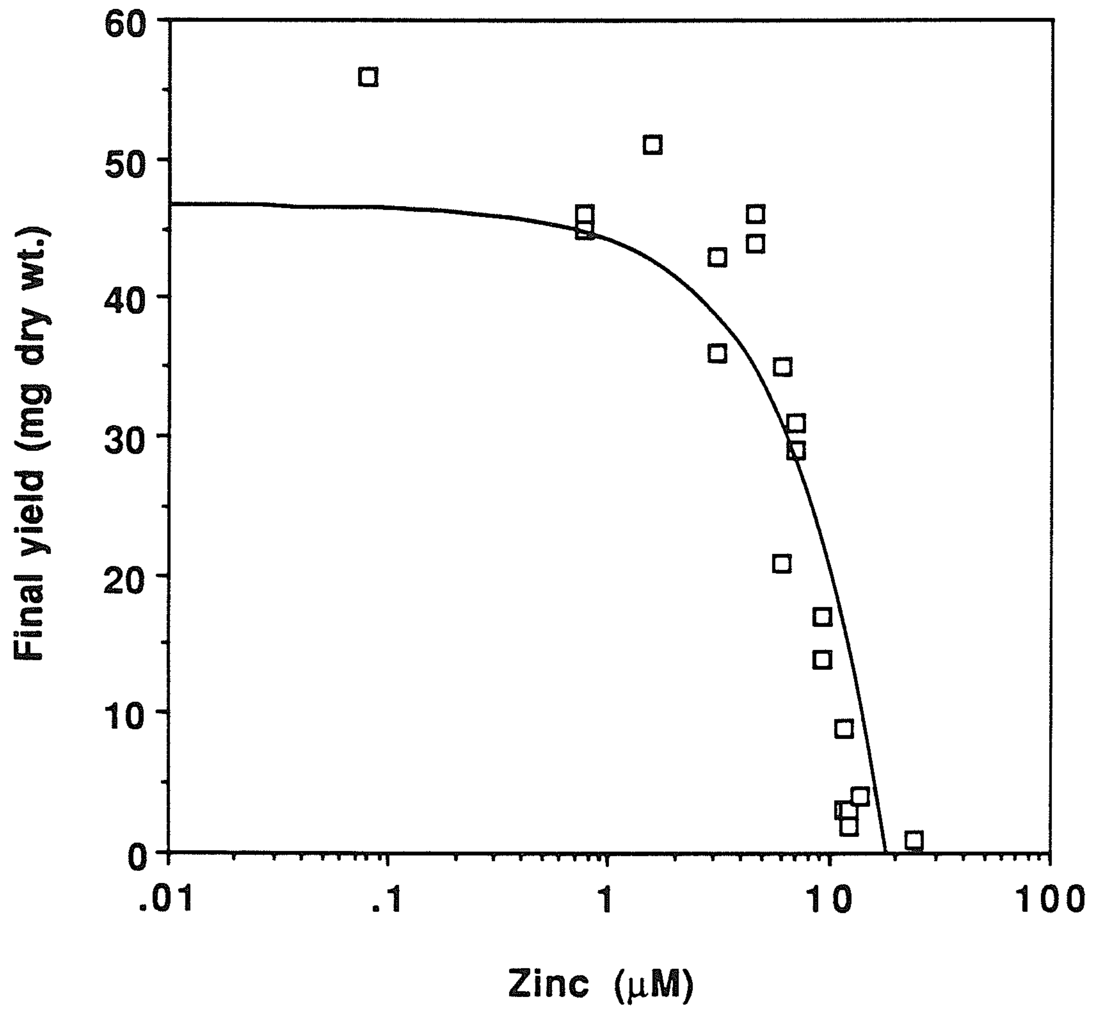
The MR responded significantly (Table 5.2) to both the external Zn and Cd concentrations. For example, the MR decreased from 182 at 0.08 μM Zn and 0 μM Cd to a low of 61 at 12.2 μM Zn and 0 μM Cd, and to 127 at 0.32 μM Cd and 0.08 μM Zn (Fig. 5.3). There was also a significant Zn by Cd interaction for the MR in L. trisulca (Table 5.2). At 0.08 μM Zn, the level of Zn used in the standard culture medium, the MR decreased a significant ($p \leq 0.01$) 12% at 0.16 μM Cd and 30% at 0.32 μM Cd (Fig. 5.3, Table 5.3). Cultures containing 3.06 and 12.2 μM Zn, however, showed no significant effects of Cd on the MR (Table 5.3), and at 3.06 μM Zn the MR in cultures with 0.16 and 0.32 μM Cd were actually higher than the corresponding cultures containing 0.08 μM Zn (Fig. 5.3). Cultures containing 6.12 μM Zn showed a significant difference in MR among the three levels of Cd at the 5% level but not at the 1% level (Table 5.2). The reduction in

Figure 5.1. The effect of the external zinc concentration on the multiplication rate (MR) in *Lemna trisulca*. $y = 175 - 5.8x$, $R^2 = 0.88$.



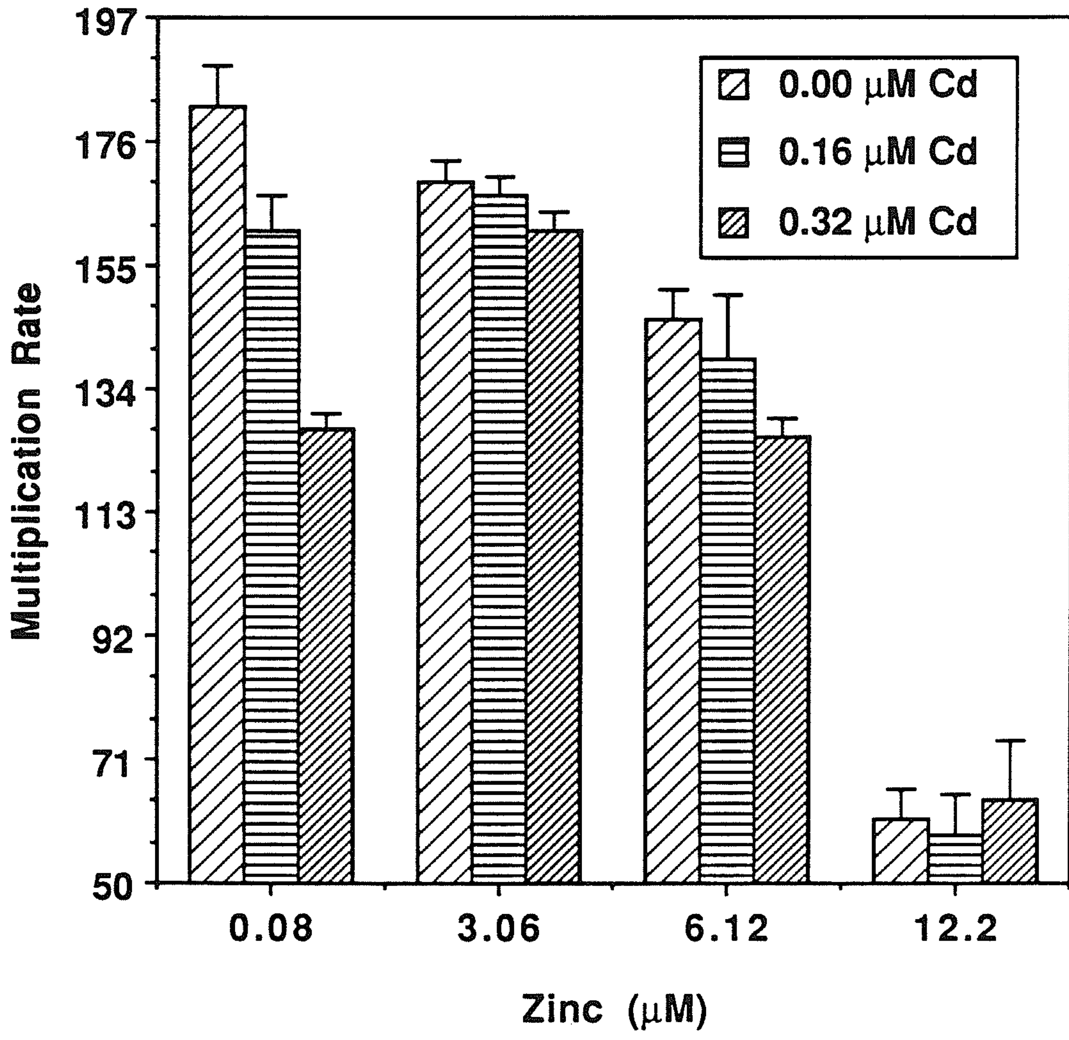
180
150
120
90
60
30
Multiplication Rate
.01
.1
1
10
100
Zinc (μM)

Figure 5.2. The effect of the external zinc concentration on final yield (mg dry wt.) in *Lemna trisulca*. $y = 49 - 2.7x$, $R^2 = 0.75$.



Final yield (mg dry wt.)
60
50
40
30
20
10
0
.01
.1
1
10
100
Zinc (μM)

Figure 5.3. The effect of the external zinc and cadmium concentration on multiplication rate in *Lemna trisulca*.



197
176
155
134
113
92
71
50
Multiplication Rate
0.00 μM Cd
0.16 μM Cd
0.32 μM Cd
0.08
3.06
6.12
12.2
Zinc (μM)

Table 5.2. Analysis of variance for multiplication rate (MR), log final yield (mg dry wt.), log frond weight (μg/frond), log Cd content (μg Cd/g dry wt.) ([Cd]) and log Zn content (μg Zn/g dry wt.) ([Zn]). The statistic MS = mean square error, F = F-ratio and P = significanc level. The model has 35 degrees of freedom; 2 for the block effect, 2 for Cd, 3 for Zn, 6 for the Zn by Cd interaction and 22 for error. The model has 23 df for [Cd] since control treatments lacking Cd were not included in the analysis; 2 for the block effect, 1 for Cd, 3 for Zn, 3 for the Zn by Cd interaction and 14 for error.

Source	Statistic	MR	Final yield	[Cd]	[Zn]	Frond weight
Cd	MS	1,210	0.162	1.06	0.054	0.0003
	F	43.4	23.2	208	11.5	0.132
	P	<0.001	<0.001	<0.001	<0.001	0.877
Zn	MS	20,300	3.76	0.446	7.80	0.097
	F	727	538	87.5	1,650	38.7
	P	<0.001	<0.001	<0.001	<0.001	<0.001
Zn x Cd	MS	500	0.034	0.010	0.026	0.014
	F	17.9	4.95	1.88	5.58	5.39
	P	<0.001	<0.001	0.179	<0.001	0.002

Table 5.3. The significance levels calculated using one-way analysis of variance at each of the four different Zn levels across all three Cd levels. The measured parameters were multiplication rate (MR), final yield (mg dry wt.) and zinc content (μg Zn/g dry wt.).

	Zinc (μM)			
Parameter	0.08	3.06	6.12	12.2
MR	<0.001	0.058	0.036	0.660
Final yield	0.002	0.237	0.031	0.673
Zn content	0.020	0.879	0.102	0.042

MR, however, was only 14% at 0.32 µM Cd and 6.12 µM Zn, compared with the 30% reduction found at 0.32 µM Cd and 0.08 µM Zn.

The multiple regression model (Table 5.4) that was developed for the MR data indicates that the Cd concentration and the square of the Zn concentration significantly reduced the MR of L. trisulca, and that Cd was more toxic than Zn. The positive coefficient for the Cd by Zn interaction term indicates that there was a significant antagonistic interaction between Cd and Zn in L. trisulca (Table 5.4). This model explained 95% of the variability in the data. The contour plot developed from the multiple regression showed that at low Zn concentrations the toxic effect of Cd was pronounced (Fig. 5.4). Increased Zn levels eliminated the Cd effect (Fig. 5.4) so that at higher Zn levels the MR at a specific Cd-Zn combination could be largely predicted by the Zn concentration alone.

The final yield in control cultures of L. trisulca was 120 ± 22 mg dry wt. (Fig. 5.5). Zinc and Cd both caused significant reductions in yield at elevated concentrations (Fig. 5.5, Table 5.2). There was also a significant Zn by Cd interaction (Table 5.2). When the external Zn was at a level of 0.08 µM, Cd reduced the final yield by 33% at 0.16 µM Cd, and 67% at 0.32 µM Cd. At 3.06 and 12.2 µM Zn, however, there was no significant decrease in the final yield as the Cd was increased from 0 to 0.32 µM (Table 5.3). Significant reductions in final yield occurred ($p \leq 0.05$) at 6.12 µM Zn as the Cd concentration rose to 0.32 µM (Table 5.3), but the reductions caused by Cd were less than at control levels of Zn. The final yield in the six treatments containing Cd at elevated Zn levels averaged approximately 1.5 times that observed in cultures grown with Cd at control levels of Zn (Fig. 5.5).

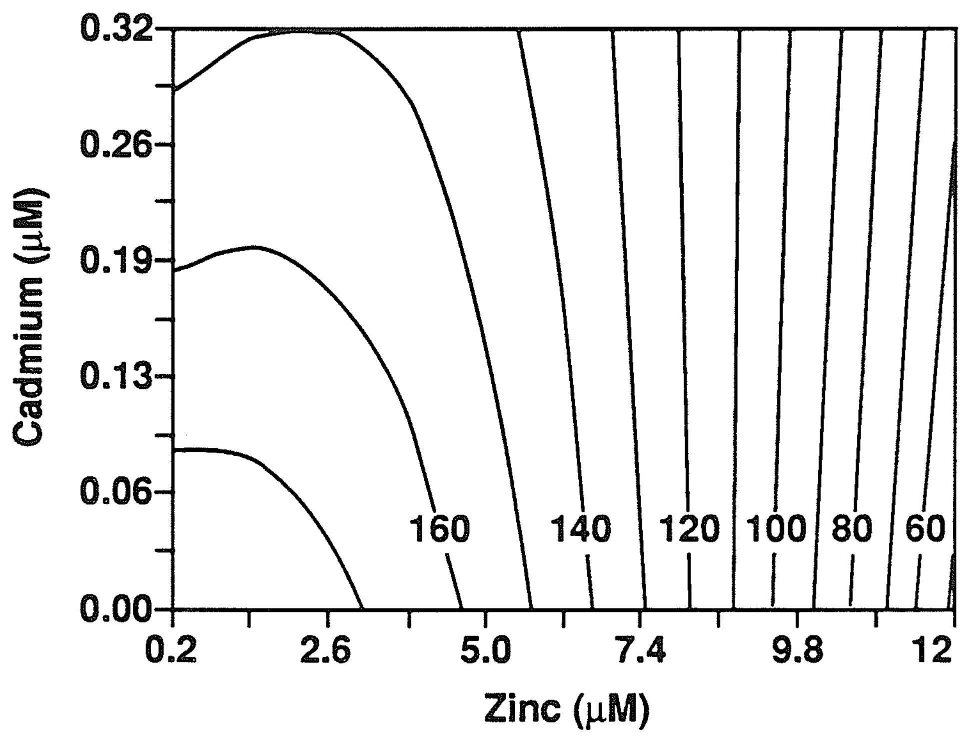
Increasing the Zn concentration from 0.08 to 6.12 µM decreased the frond weight an average of 17% but increasing the Cd concentration from 0 to 0.32 µM increased the frond weight an average of 20% (Fig. 5.6). There was a significant Zn by Cd interaction on the frond weight of L. trisulca (Table 5.2). In control cultures grown without Cd at

Table 5.4. Multiple regression model parameters calculated for the multiplication rate (MR) data. [Cd] = μM Cd, [Zn] = μM Zn, $[Cd]^2$ = μM Cd squared, $[Zn]^2$ = μM Zn squared, [Zn]*[Cd] = μM Zn times μM Cd. The model uses only those terms which proved significant. The regression model has five degrees of freedom.

Variables	Coefficient	t-test	Significance level (P)
Constant	180		
[Cd]	-100	2.24	0.033
[Zn]	0.71	0.51	0.612
$[Cd]^2$	-72	0.54	0.595
$[Zn]^2$	-0.87	8.87	<0.001
[Zn]*[Cd]	12	4.43	<0.001

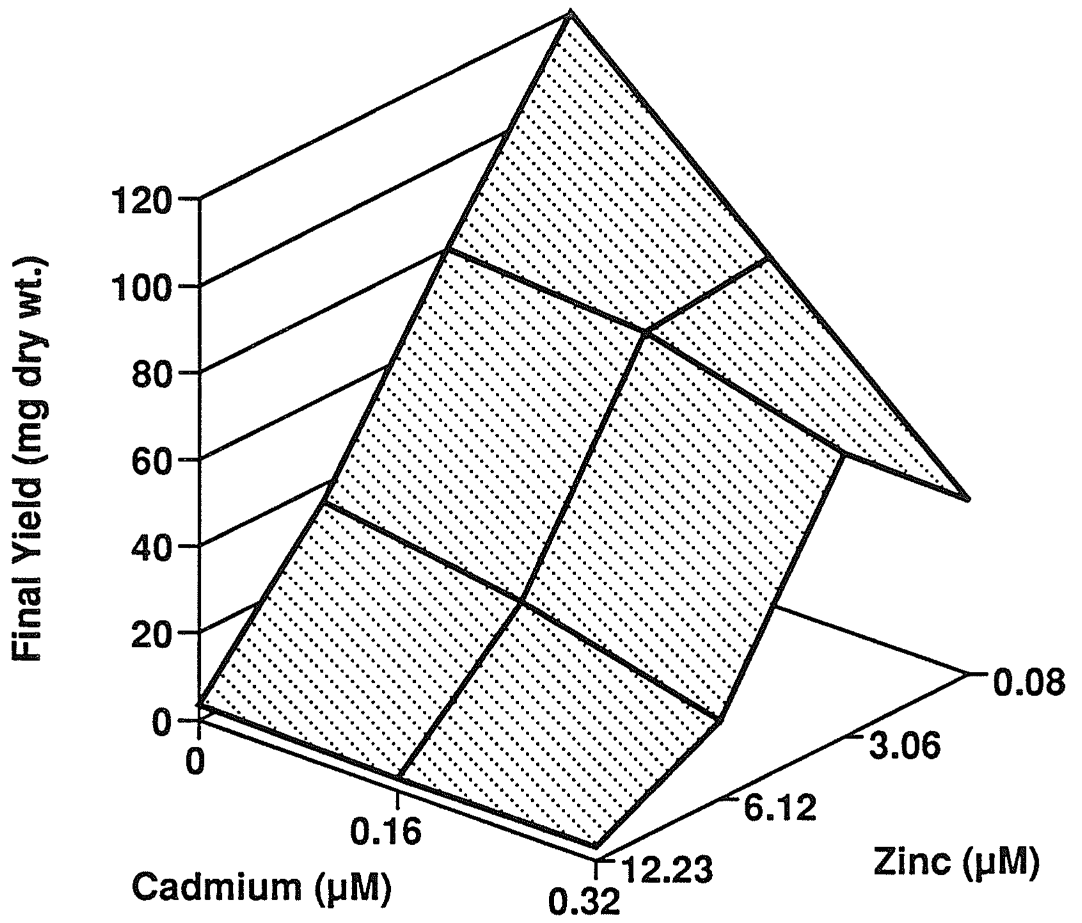
MR = 180 - 100 [Cd] - 0.87 $[Zn]^2$ + 12 [Cd]*[Zn], $R^2 = 0.95$

Figure 5.4. The contour plot for multiplication rate developed from the second order multiple regression, $y = 180 - 100[Cd] - 0.87[Zn]^2 + 12[Cd]*[Zn]$, $R^2 = 0.95$. The multiple regression is based on three cadmium and four zinc concentrations. Values within the plot are isobars for multiplication rate.



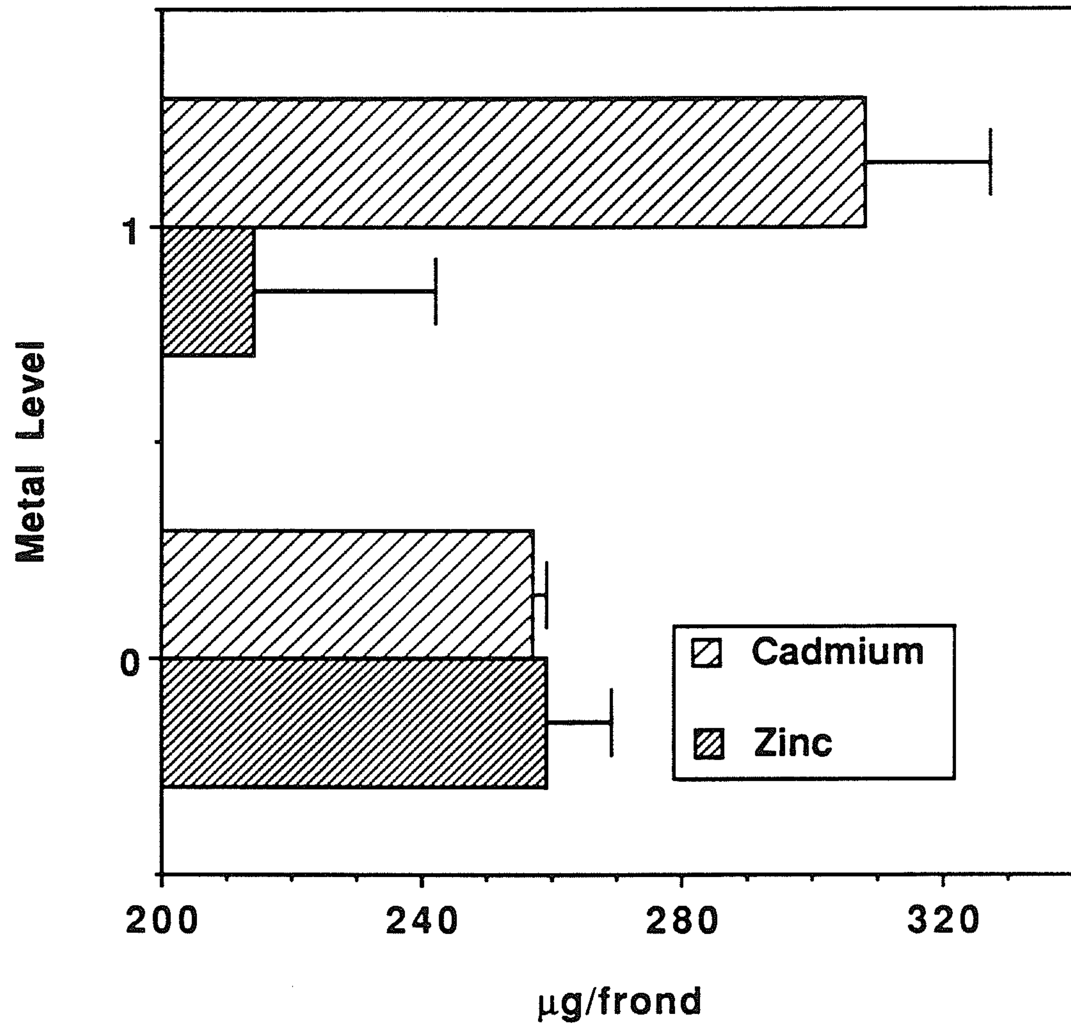
0.32
0.26
0.19
0.13
0.06
0.00
Cadmium (μM)
160
140
120
100
80
60
0.2
2.6
5.0
7.4
9.8
12
Zinc (μM)

Figure 5.5. The effect of the external zinc and cadmium concentration on final yield in *Lemna trisulca*.



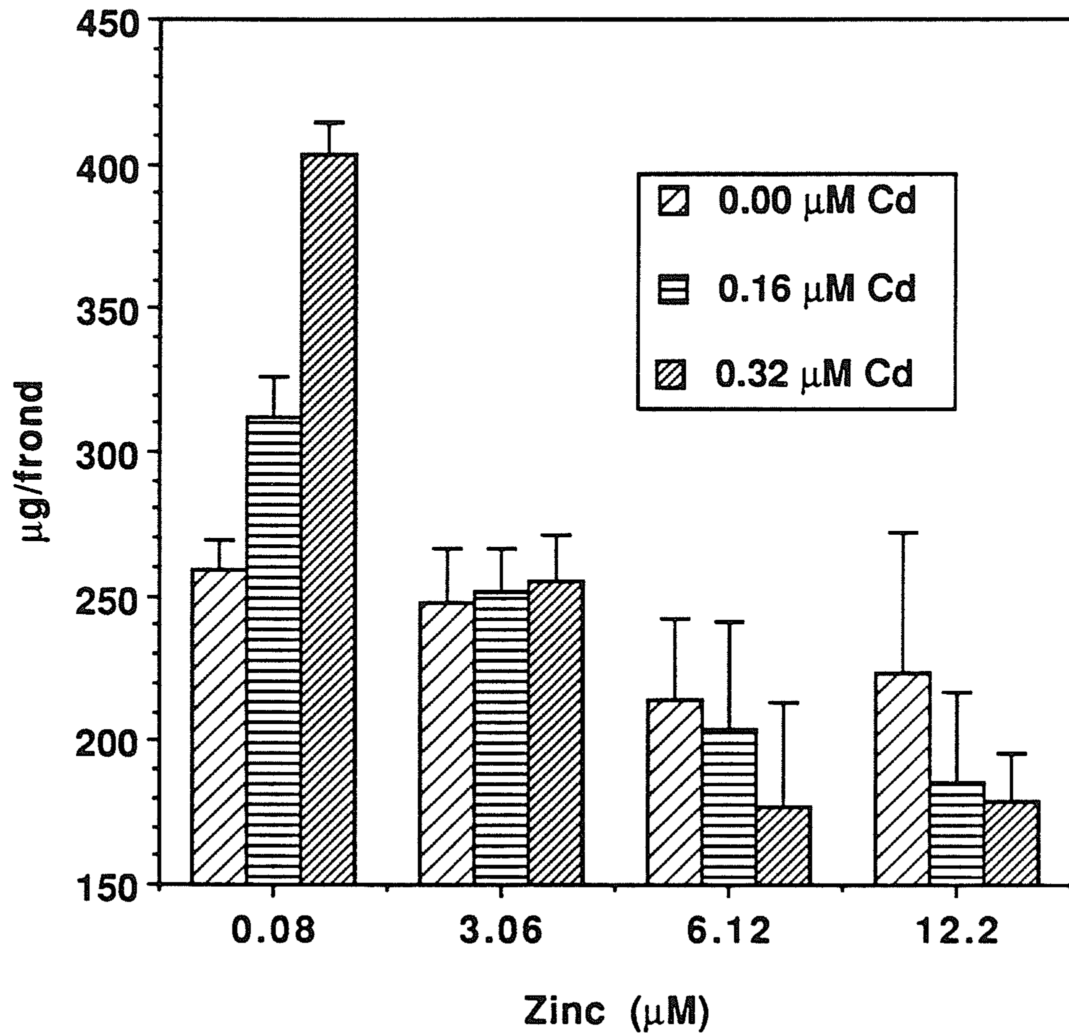
Final Yield (mg dry wt.)
120
100
80
60
40
20
0
0
0.16
0.32
Cadmium (µM)
0.08
3.06
6.12
12.23
Zinc (µM)

Figure 5.6. The effect of increased zinc or cadmium on frond weight (µg/frond) in Lemna trisulca. Metal level: 0 = 0.08 µM Zn, 0 µM Cd; 1 = 6.12 µM Zn or 0.32 µM Cd.



Metal Level
1
0
Cadmium
Zinc
200
240
280
320
μg/frond

Figure 5.7. The change in frond weight (μg/frond) in Lemna trisulca in response to increased cadmium and zinc concentrations.



450
400
350
300
250
200
150
µg/frond
0.00 µM Cd
0.16 µM Cd
0.32 µM Cd
0.08
3.06
6.12
12.2
Zinc (µM)

0.08 μM Zn, the average frond weight was 259 ± 10 μg/frond and increased to 403 ± 12 μg/frond at 0.08 μM Zn and 0.32 μM Cd (Fig. 5.7). At higher Zn concentrations, however, the trend was towards a decreased frond weight as the Cd concentration increased.

The internal Cd content of L. trisulca was significantly affected by both the external Zn and Cd concentrations (Table 5.2, Fig. 5.8). There was, however, no significant Cd by Zn interaction on the Cd content. The ANOVA for Cd content was only done on plants exposed to Cd, which reduced the degrees of freedom from 35 to 23. The Cd content of L. trisulca plants grown at 0.08 μM Zn averaged 108 ± 19 μg Cd/g dry wt. at 0.16 μM Cd and 352 ± 90 μg Cd/g dry wt. at 0.32 μM Cd. Compared with plants grown at 0.08 μM Zn, the Cd content of plants grown at 3.06 μM Zn was 39% less at 0.16 μM Cd and 57% less at 0.32 μM Cd. The Cd content of plants grown at 12.2 μM Zn, however, was 1.9 times greater at 0.16 μM Cd and 2.8 times greater at 0.32 μM Cd than the corresponding cultures grown at 0.08 μM Zn (Fig. 5.8).

The external Zn concentration significantly affected the internal Zn content (Fig. 5.9, Table 5.2). In Zn controls grown without Cd, the internal Zn content was 61 μg Zn/g dry wt. This increased to approximately 9,000 μg Zn/g dry wt. at 12.2 μM Zn. There was also a significant effect of Cd, and Zn by Cd interaction on the Zn uptake. In Zn controls, the internal content increased significantly (Table 5.3) from 61 to 143 μg Zn/g dry wt. as the Cd concentration rose to 0.32 μM. There was also a small decrease (Table 5.3) in internal Zn content at 12.2 μM Zn over the range of external Cd concentration.

Zinc concentration factors, based on the initial nominal external concentration of Zn and the dry weight of L. trisulca, ranged from 7,100 at 3.06 μM Zn to 16,000 at 6.12 μM (Table 5.5). The largest amount of Zn, totalling 230 μg, was removed from the cultures containing 6.12 μM Zn (Table 5.5). Lemna trisulca was able to remove 36% of

Figure 5.8. The effect of the external zinc concentration on cadmium content of *Lemna trisulca*.

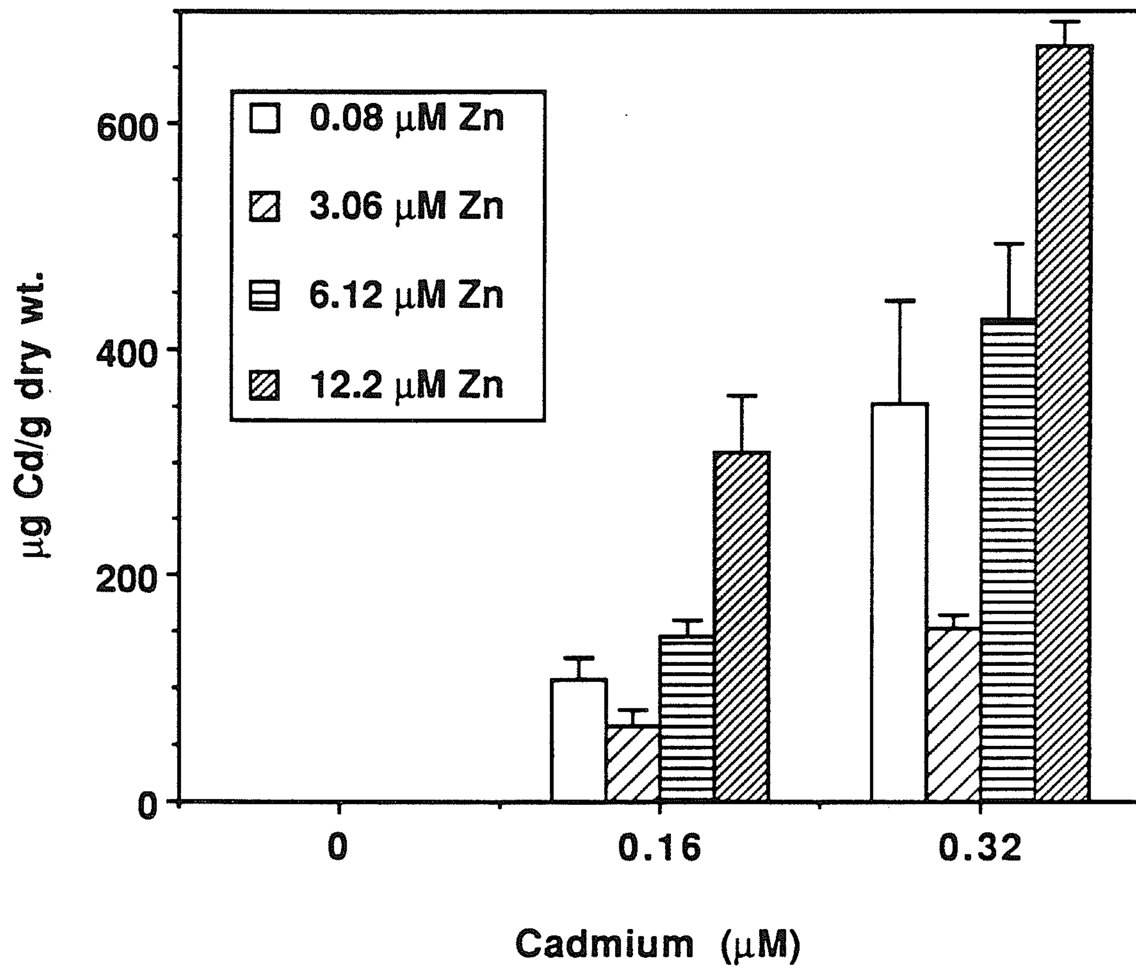
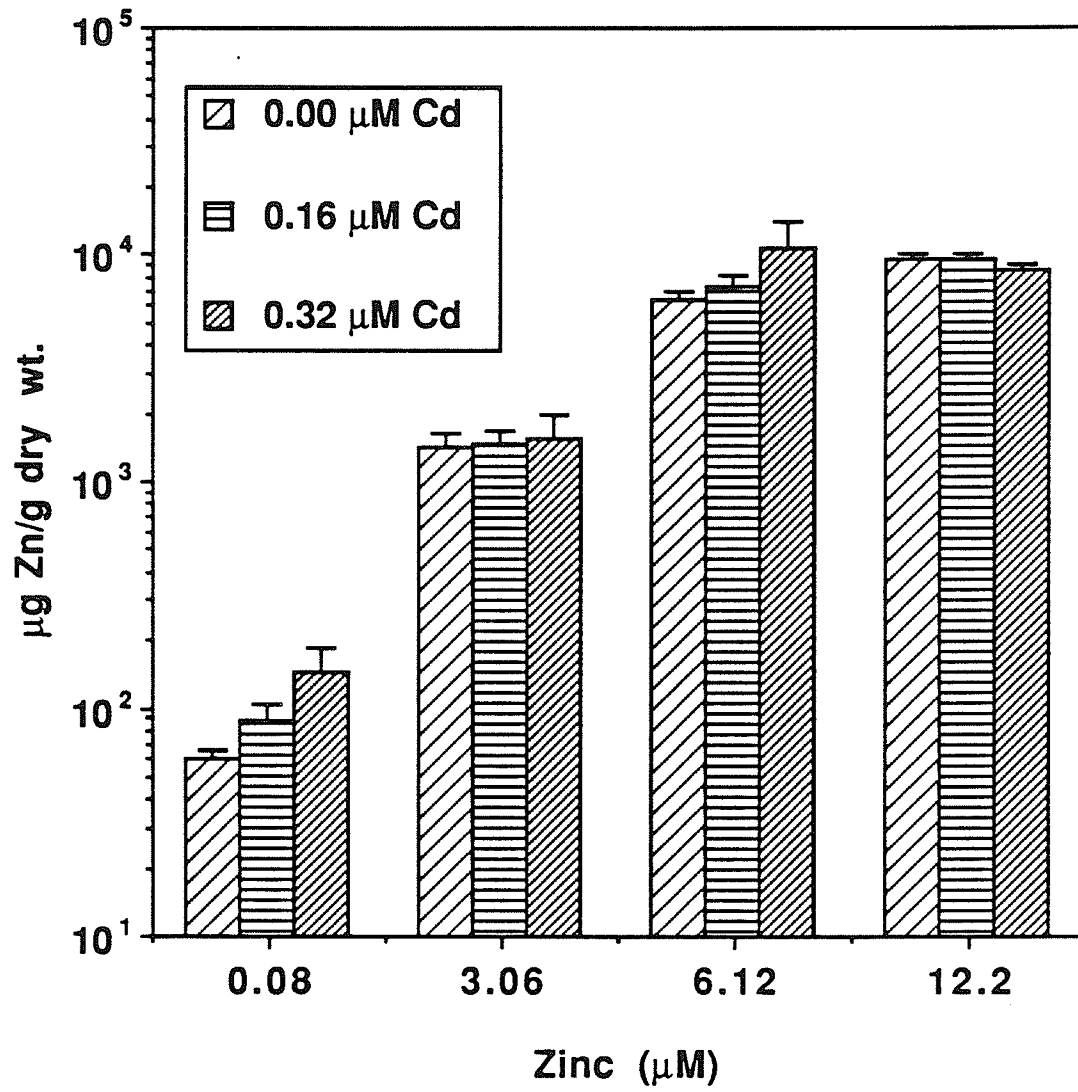

0.08 μM Zn
3.06 μM Zn
6.12 μM Zn
12.2 μM Zn
μg Cd/g dry wt.
0
200
400
600
0
0.16
0.32
Cadmium (μM)

Figure 5.9. The effect of the external zinc concentration on zinc content at three cadmium levels in _Lemna trisulca_.



0.00 μM Cd
0.16 μM Cd
0.32 μM Cd
μg Zn/g dry wt.
10^5
10^4
10^3
10^2
10^1
0.08
3.06
6.12
12.2
Zinc (μM)

Table 5.5. Characteristics of Zn uptake into Lemna trisulca under the specific conditions of this study.

Zinc (µM)	% removed[a]	Concentration Factor ($x10^3$)[b]	Total µg Zn[c] Removed
0.08	36 ± 5	12 ± 1	7.3 ± 1.0
3.06	14 ± 0	7.1 ± 1.1	110 ± 4
6.12	15 ± 2	16 ± 1	230 ± 40
12.2	1.1 ± 0.4	12 ± 1	35 ± 12

a. The amount of Cd removed from the culture medium by L. trisulca expressed as a percentage of the total available Cd in the 4 L of media.

b. Values can be converted to fresh weight concentration factors by dividing by 14.1.

c. The total amount of Cd (µg) removed by L. trisulca after 14 days growth at the specified external Cd concentrations.

the available Zn at the lowest Zn concentration. This decreased to 1.1% at 12.2 μM external Zn (Table 5.5).

DISCUSSION

Zinc decreased the MR and final yield in cultures of L. trisulca at concentrations greater than 3.0 to 4.5 μM. The EC50 for Zn was estimated at 7.8 μM for final yield and 16 μM for MR. These levels are considerably lower than results found for L. paucicostata 6746 (Nasu and Kugimoto, 1981), Spirodela polyrhiza (Schreinemakers, 1984), Azolla filiculoides Lamarck (Sela et al., 1989) and A. pinnata R. Br. (Jain and Jha, 1990), where Zn was toxic in the 75 to 150 μM range. It is possible that the greater sensitivity of L. trisulca to Zn was a result of the axenic, flow-through culture system employed in this study. The use of batch cultures has been shown to reduce the sensitivity of algae to metal toxicants (Hall, 1986) and this may also occur in higher plants. Compared with Cd toxicity (Chapter 4), Zn is approximately ten to fifteen times less toxic than Cd in L. trisulca. This is not unexpected, since Zn is an essential element for plant growth and Cd is not. In comparison to my study, Wang (1986a), examining L. minor, found Zn to be fifty times less toxic than Cd.

The Zn content of L. trisulca ranged from approximately 61 to 143 μg Zn/g dry wt. at 0.08 μM Zn, to 9,000 μg Zn/g dry wt. at 12.2 μM Zn. The concentration factors, based on dry weight, ranged from 7,100 to 16,000. These are much higher than the concentration factors of 500 to 1,000 reported for A. filiculoides (Sela et al., 1989). Zinc contents greater than 15,000 μg Zn/g dry wt. have been reported (Schreinemakers, 1984), though this was at external concentrations of up to 1,500 μM. Similar to its response to Cd, L. trisulca was most efficient at removing Zn at the lowest concentration tested, but accumulated the most Zn when the external concentration was 6.12 μM Zn.

Cadmium uptake in L. trisulca was reduced at 3.06 µM Zn compared with the uptake of Cd in cultures containing 0.08 µM Zn. Hardy and O'Keeffe (1985) and O'Keeffe et al. (1984) also found that Zn antagonized Cd uptake. At Zn concentrations of 6.12 and 12.2 µM, however, Cd uptake increased noticeably. This suggests that concentrations of Zn which are not toxic, or only marginally toxic, inhibit the uptake of Cd, perhaps by excluding Cd from binding sites in the cell wall and/or cell membrane. As Zn begins to decrease the MR, however, this mechanism appears to break down and Cd uptake actually increases. In contrast with the above results for Cd uptake, Zn at all tested concentrations, from 3.06 to 12.2 µM, mitigated the toxic action of Cd on the MR and final yield. The multiple regression model indicated a significant antagonistic interaction between Cd and Zn. Taken together, the results for the uptake and toxicity of Cd suggest that Zn, when at a much higher concentration than Cd both internally and externally, competitively excludes Cd from entering the cell at non-toxic Zn concentrations. Zinc then excludes Cd from internal binding sites as the internal Cd content increases at higher, toxic external Zn concentrations, thus mitigating Cd toxicity at all Zn levels. This interpretation suggests that Zn and Cd affect the same sites within the plant. Data on frond weight, however, do not support this. Increased levels of Cd increased the weight of L. trisulca fronds while increased Zn decreased frond weight, suggesting two different modes of action.

The results of my study are at odds with those of Hutchinson and Czyrska (1975), who found that Zn increased Cd toxicity in L. minor and Salvinia natans. This discrepancy may be due to the fact that Hutchinson and Czyrska (1975) used Zn concentrations that were insufficient for maximal growth at even the highest concentration tested. It is possible that the interaction between Zn and Cd is very different under conditions of Zn deficiency. It is also possible that determining the effects of Zn and Cd in batch cultures under non-axenic conditions (Hutchinson and Czyrska, 1975) may have confounded any interactions between Cd and Zn due to the constantly changing nutrient

and dissolved organic carbon conditions over the course of the experiment. The difference in plant species could also account for variation in the interaction between Zn and Cd, as has been found in other studies (Braek et al., 1980). It is perhaps significant, however, that the results of my study and those of Bennet and Brooks (1989), both using axenic cultures with a flow-through nutrient medium, found an antagonistic effect of Zn on Cd toxicity, even though the species tested were different.

Chapter 6. The effect of EDTA on cadmium and zinc uptake and toxicity

INTRODUCTION

The chelator ethylenediaminetetraacetic acid (EDTA) is often included in nutrient media that are used to grow aquatic macrophytes. For the culture of duckweeds, the presence of a chelator is necessary for optimal growth (Landolt and Kandeler, 1987) and consistency between replicates (Hughes, 1991) and EDTA is considered the best for duckweeds (Landolt and Kandeler, 1987). The measurement of metal toxicity, however, may be confounded by the presence of EDTA or other chelators. Metal toxicity is thought to be related to free ion activity, which is reduced by EDTA, and not to total metal concentration (Foster and Morel, 1982; Borgmann, 1983; Schreinemakers and Dorhout, 1985; Nor and Cheng, 1986).

Results on the effect of EDTA and other chelators on the uptake and toxicity of metals in aquatic macrophytes have been contradictory. The chelator EDTA antagonized the uptake and toxicity of Cu in Lemna paucicostata and L. gibba (Tanaka et al., 1982), Spirodela polyrhiza (Schreinemakers and Dorhout, 1985) and Eichornia crassipes (Nor and Cheng, 1986). Fayed and Abd-El-Shafy (1985), also using E. crassipes, found that Pb, but not Cd, uptake was reduced as the EDTA concentration was increased. Hardy and O'Keeffe (1985), also using E. crassipes, showed that chelators reduced Cd uptake and that the amount of reduction depended on the stability constant. Nasu et al. (1983), using L. paucicostata, found that EDTA antagonized the toxic effect of Cu but not that of Cd. Polar and Kucukcezzar (1986) also found chelators ineffective in ameliorating Cd toxicity in L. gibba. Kwan and Smith (1991), however, found that increasing the EDTA concentration from 6.8 to 50 μM almost completely prevented Cd uptake in L. minor. Foster and Morel (1982) showed that an increase in EDTA resulted in a dramatic recovery in growth rate and reduction in Cd uptake in the alga Thalassiosira weissflogii Grun.

The studies of Foster and Morel (1982) and Harrison and Morel (1983) indicated that the Fe concentration must be considered when examining the effect of EDTA on metal toxicity. Iron may be important because the EDTA binding constant for Fe is higher than for other cations (Skoog and West, 1969; Frank and Rau, 1990).

The purpose of this study was to determine the effect of EDTA on Cd and Zn uptake and toxicity in the submerged aquatic macrophyte L. trisulca. Experiments were carried out using aseptic technique, regular replacement of a filter-sterilized medium and defined EDTA:Fe ratios. The objective was to use my results to discuss the adequacy of the chelating properties of nutrient media which have been developed in recently published duckweed toxicity tests (U. S. EPA, 1985; Hughes, 1988; Taraldsen and Norberg-King, 1990; Wang, 1990; ASTM, 1991; Wang, 1991).

MATERIALS AND METHODS

Axenic stock cultures of Lemna trisulca were maintained in 750 ml of filter-sterilized medium contained within 1-L Erlenmeyer flasks. Stock material was grown for 6 to 8 days prior to use. For each experiment, one-frond fragments of L. trisulca were placed into 1-L Erlenmeyer flasks containing 750 ml of medium (Table 4.1) which had been filter-sterilized through 0.2 μ prewashed cellulose nitrate filters. The initial pH was 7.8 ± 0.1. This changed to 7.8 ± 0.3 by the end of the experiment since buffers could not be used because of toxic effects (Chapter 2). The range in pH changed the free ion activity of Cd^{+2} less than 5% as calculated by the speciation program MACS80 (Wagemann et al., 1990). Side-lighting was supplied continuously at 400 $\mu mol/m^2/s$ PAR by Sylvania cool-white fluorescent fixtures. The temperature was 25 ± 1°C in the growth chamber and 28 ± 2°C in the experimental flasks. Culture flasks were aerated at approximately 125 ml/min with ambient air passed through a 0.45 μ sterile filter. For experiments, L. trisulca was grown axenically for 14 days. Growth medium was replaced as follows; 50 ml on

day 4, 75 ml on day 6, 125 ml on day 7, 175 ml on day 8, 250 ml on day 9, 350 ml on day 10, 450 ml on day 11, 625 ml on day 12 and 850 ml on day 13, for a total volume of 3.7 L. This was a rate of replacement of approximately 6 ml/frond/2 day doubling time.

In Exp. 1, Cd was added at nominal concentrations of 0, 0.32 and 1.28 µM, levels which are high compared to the EC50 for Cd toxicity in L. trisulca (Chapter 4). In Exp. 1, the EDTA was added at 0, 9, 36 and 144 µM, which is high compared to natural levels (Alder et al., 1990), but within the range of values tested elsewhere (Nasu et al., 1983; Schreinemakers and Dorhout, 1985; Polar and Kucukcezzar, 1986). The standard Fe concentration was 9 µM so that the EDTA:Fe ratios were 0:1, 1:1, 4:1 or 16:1 at each of the three Cd concentrations. This resulted in a 4 by 3 factorial design of varying Cd and EDTA concentrations. A second Cd experiment was carried out where the EDTA remained constant at 9 µM and the Fe was set at 9 µM and 2.25 µM to give EDTA:Fe ratios of 1:1 and 4:1 at a constant EDTA level. In Exp. 2, Cd was tested at 0 and 1.28 µM. In Exp. 3, which tested Zn uptake and toxicity, the Fe was set at 2.25 and 9 µM at 9 µM EDTA and the EDTA was set at 36 and 144 µM at 9 µM Fe. The Zn was set at nominal concentrations of 0.08, 6.12 and 24.5 µM, levels reducing final yield and growth rate more than 50% (Chapter 5). The actual Zn and Cd concentrations were measured in the medium on day 14.

A randomized complete block design was used in which complete experiments were carried out simultaneously and then repeated at later dates to generate a total of three replicates per treatment. This was necessary to ensure that changes in the growth rate of control cultures over time did not confound the measurement of Cd and Zn toxicity.

Plants were removed from the culture flasks after 14 days and the fronds were counted. The multiplication rate (MR) was measured on frond production between day 2 and day 14 (Chapter 7) and was defined as:

$$MR = 1000(\text{Log } F_t - \text{Log } F_o)/t$$

where F_o is the initial frond number, F_t is the final frond number, and t is the culture time in days (Hillman, 1961). The time in days needed to double the number of fronds equals 301/MR. Plants were washed three times in distilled water and dried at 85°C for 24 h to determine final yield in mg oven dry wt. Concentrated HNO_3 was added and heated to dryness four times, after which the material was dissolved in heated 5N HCl, filtered with Whatman # 42 ash-free filter papers and diluted to 100 ml with deionized water. Zinc was determined by atomic absorption spectroscopy and Cd with a Varian GTA-95 graphite furnace connected to a Varian AA-975 atomic absorption spectrophotometer. Background correction and an injection volume of 15 µl was used. The precision of the Cd measurement, based on 24 duplicate measurements over the range of absobance, was 4.4 ± 5.7% (as coefficient of variation). On spiked plant samples, the recovery of Cd was 114 ± 7%, with a coefficient of variation of 5.9%. All reagents were ACS grade or better. Glassware was acid washed in 3N HNO_3 and rinsed repeatedly with distilled, deionized water. The water used throughout was distilled and then deionized with a final conductivity of approximately 0.06 µS/cm. Data from this chapter are contained in Tables A.5.1, 2, 3 in Appendix A. This chapter was published as Huebert and Shay (1992b).

RESULTS

External Cd and Zn concentrations

The actual external metal concentrations were measured on the last day of experiments (Table 6.1). The precision of the measurement (as coefficient of variation) averaged 8.8% for Cd and 5.0% for Zn. The measured metal concentrations averaged 98% of the nominal concentration for Cd and 99% of the nominal concentration for Zn. Only three values were less than 90% of the target concentration. The data indicate that the schedule used for replacement of medium in this study was adequate for maintaining the target metal concentrations.

Table 6.1. The external Cd and Zn concentrations (μM) as measured in the culture flasks on the last day of the experiment.

Metal	EDTA (μM)				
(μM)	0	9	9	36	144
Cadmium					
0	0[a]	0	0	0	0
0.32	0.33 ± 0.03	0.34 ± 0.06		0.33 ± 0.04	0.30 ± 0.05
1.28	1.23 ± 0.05	1.35 ± 0.05	1.32 ± 0.02	1.15 ± 0.09	1.09 ± 0.07
Zinc					
6.21		5.50 ± 0.30	5.96 ± 0.30	6.27 ± 0.15	6.27 ± 0.15
24.5		19.9 ± 1.5	24.5 ± 1.5	27.5 ± 1.5	27.5 ± 1.5

a. The value zero denotes concentrations below the detection limit for Cd.

The effect of EDTA on Cd uptake and toxicity (Exp. 1)

The interaction between the EDTA concentration and Cd toxicity was measured on MR (Fig. 6.1) and final yield (Table 6.2). For all experiments, the standard medium contained no Cd, 0.08 μM Zn and 9 μM EDTA at a 1:1 EDTA:Fe ratio. A control grown under these conditions was included in each experimental block.

In the control treatment of Exp. 1, final yield was 119 ± 46 mg dry wt. and MR was 187 ± 15, which is a doubling time of 1.6 days. There was a significant difference in the replicates ($F = 43$, $df = 2$, $p < 0.001$) so the MR (Fig. 6.1) and final yield (Table 6.2) were presented not as values but as a ratio of treatment to control.

The interaction between EDTA and Cd uptake is shown in Table 6.2. Plants grown under otherwise standard conditions had a Cd content of 620 ± 350 μg Cd/g dry wt. when 0.32 μM Cd was added, and 2,300 ± 1,000 μg Cd/g dry wt. when the medium contained 1.28 μM Cd (Table 6.2). Cadmium accumulations were even higher when EDTA was removed from the medium. In treatments containing elevated EDTA levels, the uptake of Cd was reduced by 98 to approximately 100% at even the highest Cd concentration tested (Table 6.2).

When 0.32 μM Cd was added to the standard medium, the MR was decreased by 35% and the final yield was decreased by 83%. Cadmium added to the standard medium at a concentration of 1.28 μM decreased the MR by 74% and the final yield by 97% (Fig. 6.1, Table 6.2). There was little change in the toxicity of Cd to L. trisulca when EDTA was completely removed from the medium. With no EDTA in the medium, however, there was considerable precipitate, and even with no Cd added, the plants appeared stunted and chlorotic, with the MR only 72% of that measured in control cultures (Fig. 6.1). The average frond weight also decreased, from 270 ± 10 μg dry wt./frond in control treatments, to 220 ± 20 μg dry wt./frond in cultures lacking EDTA. In treatments where the medium contained 36 or 144 μM EDTA, there was no change in

Figure 6.1. The effect of EDTA on Cd toxicity as measured by the ratio between the treatment and control for multiplication rate in Lemna trisulca. The Fe was 9 µM in all treatments. * designates the control treatment.

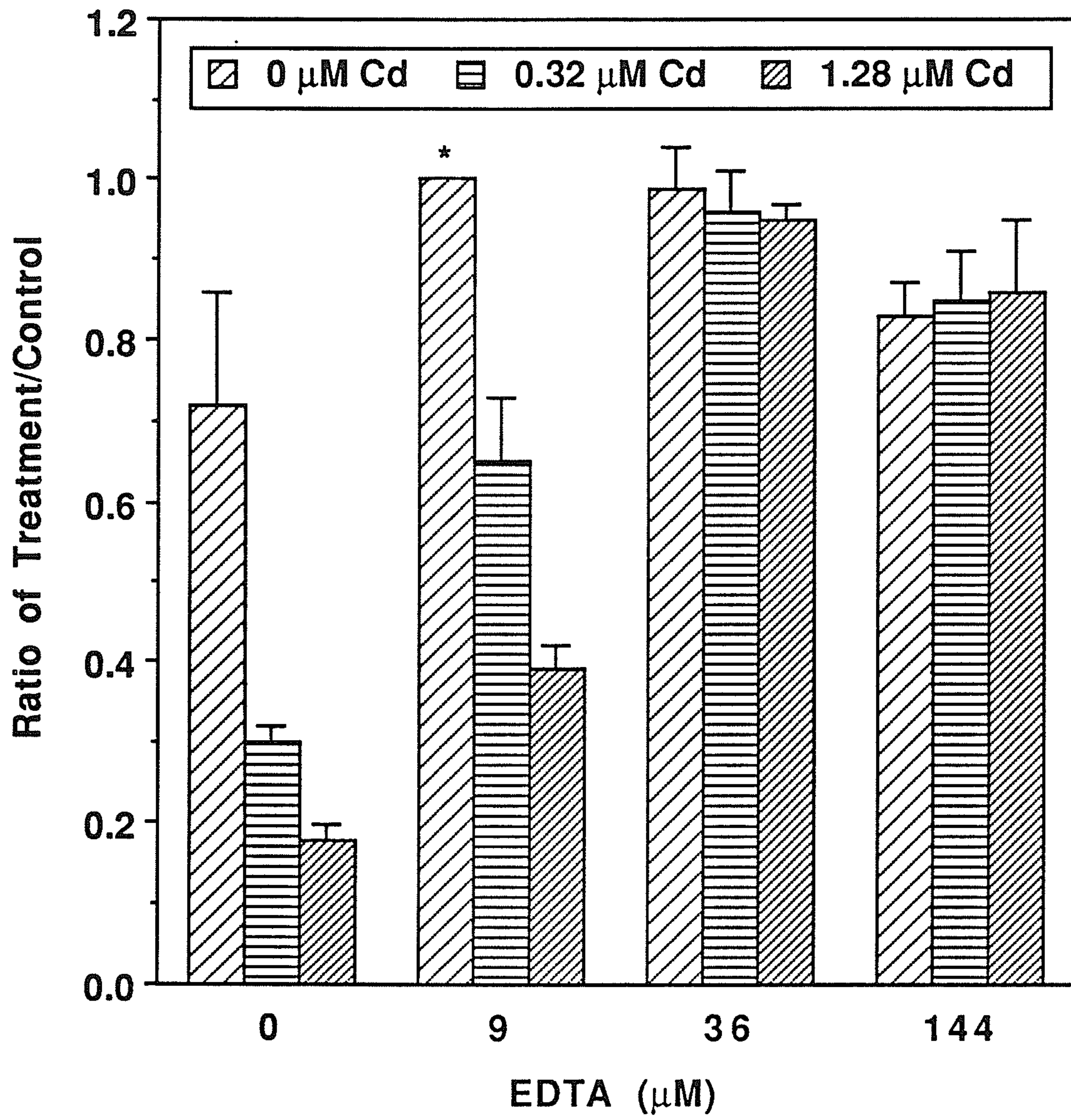

0 μM Cd
0.32 μM Cd
1.28 μM Cd
Ratio of Treatment/Control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
0
9
36
144
EDTA (μM)

Table 6.2. The Cd content (µg Cd/g dry wt.) and ratio between treatment and control for the final yield (mg dry wt.) of Lemna trisulca in response to external Cd and EDTA concentrations. The Fe concentration was 9 µM for all treatments.

Cadmium	EDTA (µM)			
(µM)	0	9	36	144
Treatment/control[a]				
0	0.21 ± 0.23	1.00	0.96 ± 0.19	0.47 ± 0.05
0.32	0.01 ± 0	0.17 ± 0.04	0.82 ± 0.18	0.52 ± 0.14
1.28	0.01 ± 0.01	0.03 ± 0.01	0.82 ± 0.06	0.55 ± 0.23
Cd Content				
0	1.6 ± 0.9	1.2 ± 1.5	0[b]	0.3 ± 0.6
0.32	3,000 ± 650	620 ± 350	11 ± 4	1.3 ± 0.7
1.28	2,500 ± 1200	2,300 ± 1000	51 ± 4	5.8 ± 3.0

a. Values for final yield in the Cd experiment are expressed as a percentage of a control value due to significant differences between the replicates, which had been blocked for time.

b. The value zero denotes a Cd content below the detection limit.

the MR or final yield of L. trisulca when Cd was added (Fig. 6.1, Table 6.2). The highest EDTA concentration tested, however, did reduce the MR by 17% and the final yield by 53%.

The effect of Fe on Cd uptake and toxicity (Exp. 2)

The uptake of Cd is compared with the final yield at different Fe concentrations in Table 6.3. Figure 6.2 shows the interaction between the Fe concentration and Cd toxicity. Lemna trisulca grown under otherwise standard conditions in the presence of 1.28 μM Cd accumulated 3,400 ± 1,000 μg Cd/g dry wt. and produced only 2.2 mg dry wt. in 14 days growth. This was a reduction in the MR of 76% (Fig. 6.2). Reducing the Fe concentration to 2.25 μM increased the EDTA:Fe ratio to 4:1. Under these conditions, Cd uptake at 1.28 μM Cd was reduced by 97% and Cd toxicity was completely antagonized (Table 6.3, Fig. 6.2).

The effect of EDTA and Fe on Zn uptake and toxicity (Exp. 3)

The Zn content of L. trisulca in response to the external EDTA and Fe concentration is shown in Table 6.4. Plants grown at 0.08 μM Zn contained 0.07 to 0.15 mg Zn/g dry wt. regardless of the EDTA concentration. Under otherwise standard conditions, this increased to 8.7 ± 2.9 mg Zn/g dry wt. at 6.12 μM Zn, and approximately 20 mg Zn/g dry wt. at 24.5 μM Zn. Increasing the EDTA:Fe ratio to 4:1 by decreasing the Fe concentration had little effect on Zn content, but elevated EDTA levels reduced uptake by up to 98% (Table 6.4).

The interaction between EDTA, Fe concentration and Zn toxicity was measured by MR (Fig. 6.3) and final yield (Table 6.4). In the control treatment, the MR was 153 ± 7, which is a doubling time of 2.0 days, and the final yield was 54 ± 14 mg dry wt. When 6.12 μM Zn was added to the standard medium, the MR decreased 28% and the final yield decreased 72%. Zinc at a concentration of 24.5 μM in an otherwise standard

Table 6.3. The internal Cd content (μg Cd/g dry wt.) and final yield (mg dry wt.) in Lemna trisulca in response to external Cd and Fe concentrations at 9 μM EDTA. At each Cd concentration, the top number is the Cd content and the bottom value is the final yield.

Cadmium	Iron (μM)	
(μM)	2.25 (4:1)[a]	9.0 (1:1)
0	0[b]	0
	58 ± 6	60 ± 4
1.28	106 ± 9	3,400 ± 500
	65 ± 8	2.2 ± 0.4

a. The numbers in brackets are the EDTA:Fe ratios at that Fe concentration.

b. Indicates values below the detection limit.

Table 6.4. The final yield (mg dry wt.) and Zn content (mg Zn/g dry wt.) of Lemna trisulca in response to the external Zn, Fe and EDTA concentration.

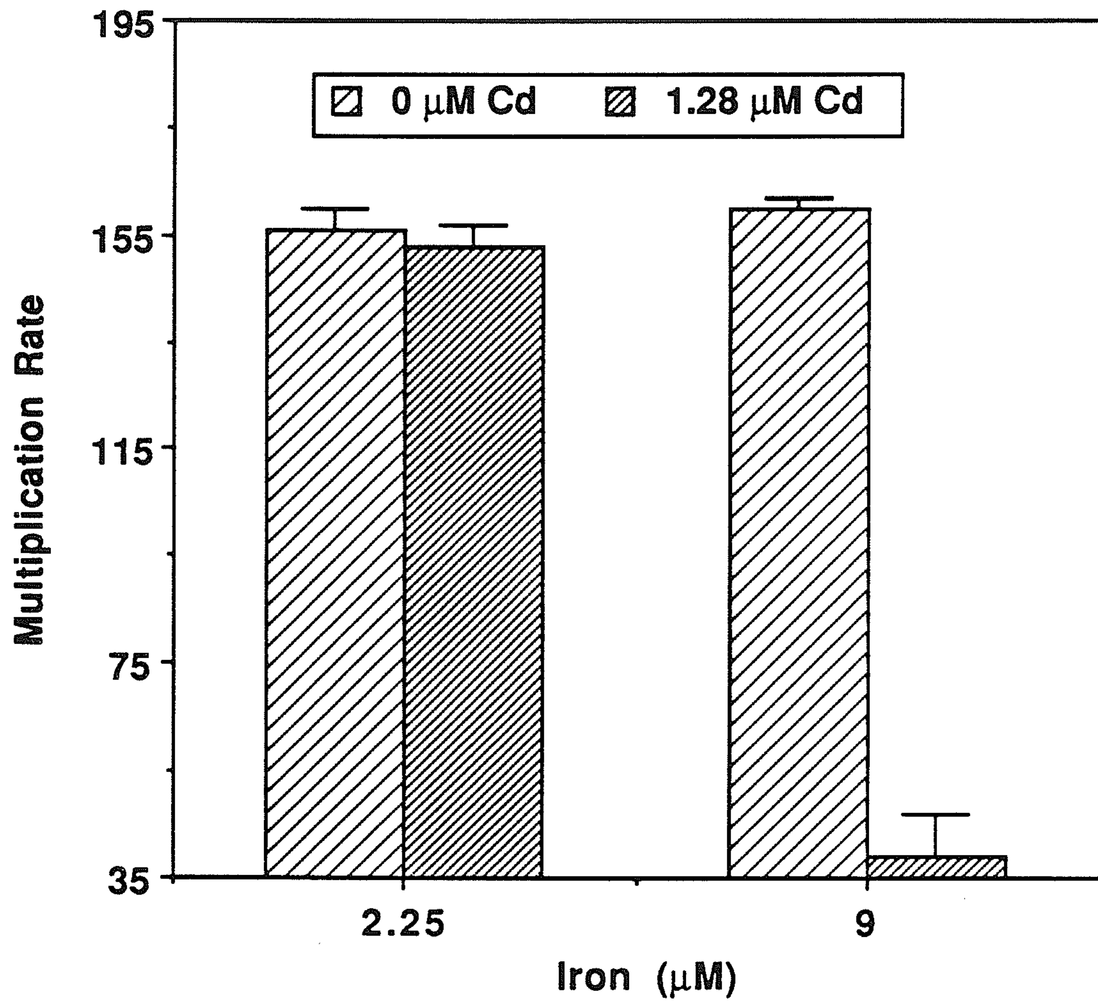
Zinc	EDTA (µM)			
(µM)	9 (1:1)[a]	9 (4:1)	36 (4:1)	144 (16:1)
Final Yield				
0.08	54 ± 14	55 ± 3	57 ± 6	19 ± 2
6.12	15 ± 9	21 ± 12	55 ± 9	16 ± 8
24.5	0.6 ± 0.1	0.6 ± 0.1	27 ± 12	27 ± 15
Zn Content				
0.08	0.13 ± 0.03	0.07 ± 0.02	0.07 ± 0.07	0.15 ± 0.10
6.12	8.7 ± 2.9	6.6 ± 2.1[b]	0.32 ± 0.10	0.41 ± 0.19
24.5	20[c]	30[c]	8.2 ± 3.8	0.34 ± 0.07

a. The numbers in brackets denote the EDTA:Fe ratio at that chelator concentration.

b. Sample size equals two for this treatment.

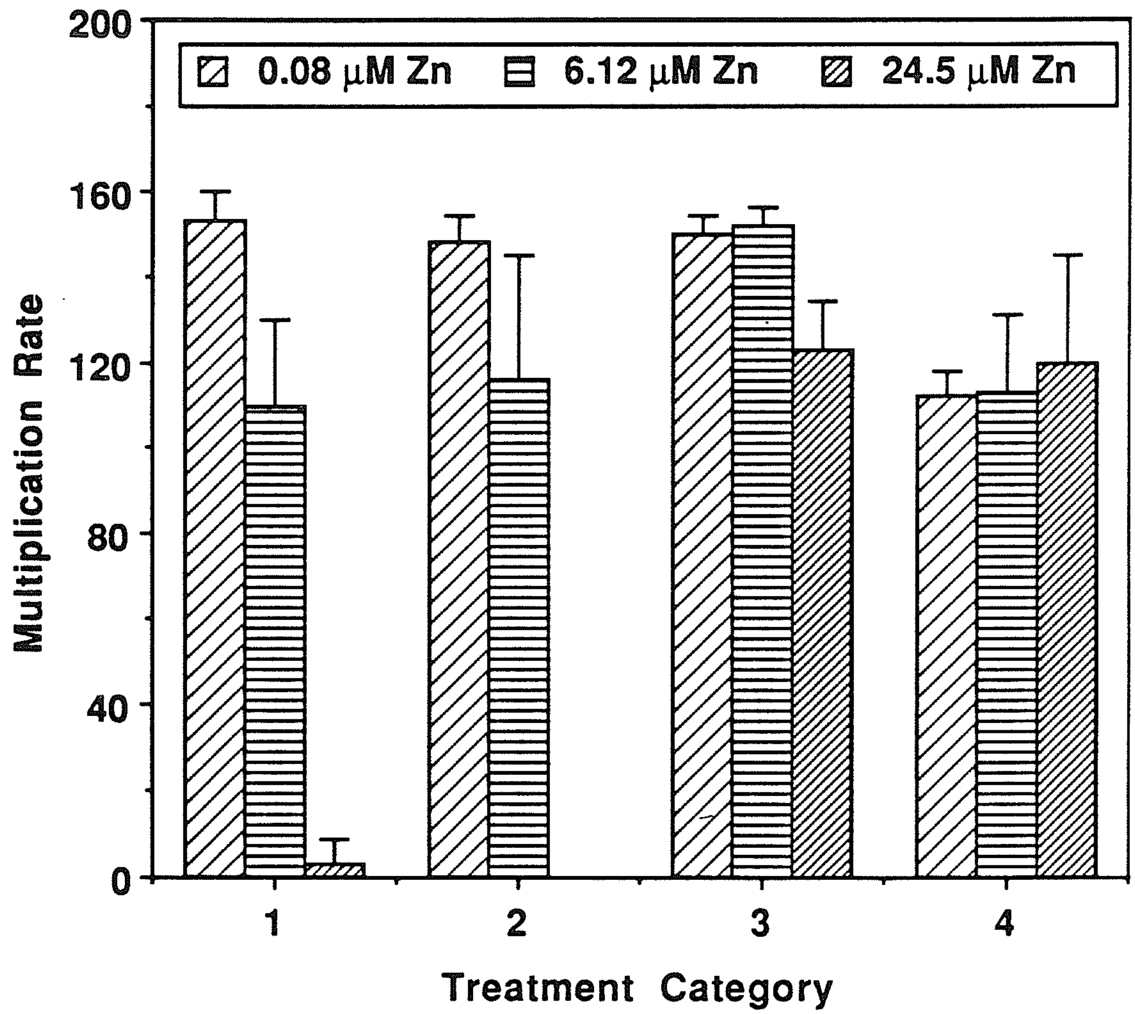
c. Sample size equals one for these treatments due to contamination of digests and subsequent loss of samples.

Figure 6.2 The effect of Fe on Cd toxicity as measured by the multiplication rate of Lemna trisulca. The EDTA concentration was 9 μM in all treatments.



0 μM Cd
1.28 μM Cd
195
155
115
75
35
Multiplication Rate
2.25
9
Iron (μM)

Figure 6.3. The effect of the EDTA:Fe ratio on Zn toxicity as measured by the multiplication rate of Lemna trisulca. Treatment 1; 9µM EDTA at a 1:1 EDTA:Fe ratio, 2; 9 µM EDTA at a 4:1 EDTA:Fe ratio, 3; 36 µM EDTA at a 4:1 EDTA:Fe ratio, 4; 144 µM EDTA at a 16:1 EDTA:Fe ratio.



0.08 μM Zn
6.12 μM Zn
24.5 μM Zn
200
160
120
80
40
0
Multiplication Rate
1
2
3
4
Treatment Category

medium decreased the MR and final yield 98 to 99%. There was little change in Zn toxicity when the EDTA:Fe ratio was increased to 4:1 by reducing the Fe concentration to 2.25 μM (Table 6.4, Fig. 6.3). At elevated EDTA levels, however, there was almost no toxicity at even 24.5 μM Zn. As in Exp. 1, EDTA at a concentration of 144 μM reduced growth, by 27% for MR and 65% for final yield.

DISCUSSION

The effect of EDTA on Cd and Zn toxicity

For L. trisulca, when the amount of available EDTA exceeded the concentration of Cd or Zn, the chelator EDTA antagonized Cd and Zn toxicity, and reduced uptake by more than 95%. The antagonistic effect of EDTA suggests that in L. trisulca it is the free ion activity and not the total concentration which determines the uptake and toxicity of Cd and Zn. This is consistent with the conclusions of Schreinemakers and Dorhout (1985), who found that increasing the EDTA concentration decreased Cu toxicity. They concluded that in S. polyrhiza a concentration of 12 μM free Cu was needed to inhibit growth. Nor and Cheng (1986) suggested that an EDTA:Cu ratio of 3.0 was necessary to prevent the uptake of Cu in E. crassipes. Tanaka et al. (1982) found that 30 μM EDTA eliminated the accumulation of Cu at concentrations as high as 20 μM Cu in L. paucicostata and 4 μM Cu in L. gibba. For L. minor, 50 μM EDTA completely prevented the uptake of Cd (Kwan and Smith, 1991). Chelators also antagonize metal toxicity in algae (see Foster and Morel, 1982) and higher organisms (Borgmann, 1983). In contrast to these results, other studies found EDTA had no effect on Cd uptake and toxicity in L. paucicostata (Nasu et al., 1983), E. crassipes (Fayed and Abd-El-Shafy, 1985) and L. gibba (Polar and Kucukcezzar, 1986).

The experiments reported here used axenic cultures and a filter-sterilized medium, a portion of which was regularly replaced during experiments. Studies using either

replacement of media (Kwan and Smith, 1991) or a defined, filter-sterilized medium (Foster and Morel, 1982) produced similar results. Inconsistencies in the effect of EDTA may perhaps be explained by differences in methods. Most studies examining metal toxicity in aquatic macrophytes have used either non-axenic or autoclaved media, and static cultures with small volume:plant ratios. This may lead to problems, since EDTA breaks down over time due to photo-oxidation (Lockhart and Blakely, 1975; Alder et al., 1990; Frank and Rau, 1990), and autoclaving almost completely destroys EDTA (A. L. McIlraith, pers. comm.).

In this study, EDTA reduced Zn uptake at a higher concentration than it did for Cd. The Zn concentrations, however, were approximately twenty times higher. When EDTA was in excess of the Zn concentration, Zn toxicity was as effectively neutralized as Cd. There did not seem to be a difference in the effect of excess EDTA on the uptake and toxicity of an essential vs non-essential element. Nasu et al. (1983), in contrast, found that EDTA had little or no effect on toxicity of Cd. The uptake of Cd decreased to negligible levels only as the chelator concentration was increased to 400 µM. Copper toxicity, however, was antagonized by 30 µM EDTA in L. paucicostata, apparently by the almost complete prevention of Cu absorption. This result is counter-intuitive, since one would expect that an essential element would be taken up more readily and tenaciously than a non-essential one such as Cd. The paradox is that EDTA increases the availability of Fe (see Anderson and Morel, 1982), while at the same time sharply reducing the uptake of other metals such as Cu, Cd or Zn.

The effect of Fe on Cd and Zn toxicity

Decreasing the Fe concentration from 9 to 2.25 µM in the presence of 9 µM EDTA caused a 97% reduction in Cd uptake and antagonized the toxicity of 1.28 µM Cd. This suggests that the amount of EDTA available for chelating Cd or Zn is controlled by the Fe concentration, which is not unexpected since Fe has the highest EDTA binding

constant of any metal (Skoog and West, 1969). Frank and Rau (1990) have estimated that most of the EDTA found in surface waters should also be present as Fe complexes. This suggests that the mobilization of metals by EDTA in natural systems (Alder et al., 1990) depends on whether EDTA is present in excess of the Fe concentration, and therefore available.

In contrast, Foster and Morel (1982) found that only when Fe was increased, was excess EDTA effective in ameliorating Cd toxicity in the alga Thallassiosira weissflogii. They concluded that there was a physiological interaction between Fe and Cd, and in a later study showed that Cd inhibited Fe uptake (Harrison and Morel, 1983). Their study differed from mine in that EDTA was present at 10 or 100 μM and Fe was added at 0.02 to 2.0 μM, so that EDTA was always in excess of the Fe concentration. It is possible that a physiological interaction between Cd and Fe would also be found in higher plants under these conditions.

Implications for toxicity assessment

Several protocols have recently been published which use duckweeds for toxicity assessment (U. S. EPA, 1985; Hughes, 1988; Taraldsen and Norberg-King, 1990; Wang, 1990; ASTM, 1991; Wang, 1991). The media described in these protocols contain various amounts of chelator. The concentration of chelator is critical to the accuracy of the protocol, since my data and other studies (Foster and Morel, 1982; Kwan and Smith, 1991) have shown that the presence of a chelator in growth media will alter metal uptake and toxicity due to metal-chelator interactions.

The U. S. EPA (1985) has entirely removed chelators from their medium to prevent their results being confounded by metal-chelator interactions. This is inappropriate, however, since without EDTA, the growth of the duckweed L. trisulca is poor and precipitation occurs in the medium. Chelators such as EDTA must be included in nutrient media to optimize growth (Landolt and Kandeler, 1987) and consistency between

replicates (Hughes, 1991). It is also inappropriate to use a soil extract to replace EDTA (Taraldsen and Norberg-King, 1990), since soil extracts will chelate metals a variable and unknown amount (Garvey et al., 1991). No confidence can be placed in the results of experiments examining metal uptake and toxicity if the chelating properties of the medium are unknown.

There are two procedures that may effectively deal with the chelating requirements of aquatic macrophytes without confounding the measurement of uptake and toxicity of metals. The first involves maintaining a 1:1 chelator:Fe ratio at as low a concentration as will support maximum multiplication rates. This will minimize metal chelation and was used for the present study. The EDTA/Fe stock solution must be filter-sterilized and added after autoclaving when a low chelator concentration is used since, as discussed previously, autoclaving almost completely destroys EDTA. The problem with using a low EDTA concentration at a 1:1 EDTA:Fe ratio is that EDTA is unstable in light (Lockhart and Blakely, 1975) and plants take up Fe. Thus there are unknown changes over time in the chelating properties of the medium. It is imperative, therefore, that replacement of media occurs to minimize these changes (Rand and Petrocelli, 1985).

The second procedure is to maintain a chelator concentration in excess of the Fe concentration and of the metals in the medium, so that changes in metal chelation will be minimized (Foster and Morel, 1982; Harrison and Morel, 1983). Toxicity can then be expressed on the basis of the free metal ion. It is inadequate, however, to add chelator in excess of the Fe concentration but not in excess of the metal toxicants (as in Wang, 1990; ASTM, 1991). Under these conditions, metal speciation will change dramatically when the EDTA is saturated and toxicity will depend more on the EDTA concentration than the metal concentration. Adding excess chelator also requires a speciation computer program in order to calculate free ion activities. These data are often difficult to obtain and of uncertain value without precisely defined conditions. Another problem is that high chelator concentrations may be inhibitory, as found in my study where 144 µM EDTA

decreased the MR by 17 to 27%. Finally, chelated metals may be toxic in themselves (Borgmann, 1983).

It may be impossible to define or control interactions between a basal medium and a metal toxicant (Metaxas and Lewis, 1991). To avoid the problems associated with determining ion activities, perhaps the internal metal load could be related to reductions in growth rate instead of an external concentration (Borgmann et al., 1991; Chapter 4). This is not without problems, however, as it is possible that the effect of the internal metal content could vary with length of exposure and internal nutritional factors and lack accuracy due to different washing procedures.

Chapter 7. The response of Lemna trisulca to cadmium

INTRODUCTION

There is little agreement regarding the optimum length of time for toxicity tests using duckweeds. Landolt and Kandeler (1987) state that duckweeds should be grown for four weeks under constant conditions before any growth rate measurements are taken, but toxicity tests that use duckweeds are only of 4 to 7 days duration (U. S. EPA, 1985; Hughes et al., 1988; Taraldsen and Norberg-King, 1990; Wang, 1990; ASTM, 1991; Wang, 1991). Kwan and Smith (1991), using Lemna minor, showed that the Cd content reached 'steady state' conditions after approximately 12 days when the plant was exposed to 1.5 µM Cd. This suggests that perhaps four weeks is too long but that 4 to 7 days is not long enough to adequately assess toxicity. Outridge and Hutchinson (1991) and Outridge et al. (1991), using Salvinia spp., implied that the response to Cd will change depending on the length of exposure. They showed that phytochelatins increased in response to Cd and have suggested that induction of Cd tolerance occurs in Salvinia spp. If not considered, this process could compromise the assessment of toxicity.

Studies have found that the intrinsic growth rates of aquatic plants are not constant over time (Huebert and Gorham, 1983; Landolt and Kandeler, 1987; Thorsteinsson et al., 1987; Wang, 1990). Wang (1990), for instance, found that the doubling time of L. minor varied from 1.3 to 2.8 days over an 18 month period. Data on the effect of a reference toxicant over time are scarcer. Wang (1990) reported no cyclic changes in the effect of Cr on L. minor, but unfortunately did not examine the relationship between intrinsic growth rate and the Cr reference toxicant. Fluctuations or relationships between these two factors could confound efforts to assess toxicity by seasonal changes in the effect of a toxicant.

There have been few studies examining changes in the growth strategy of aquatic macrophytes in response to stress (Room, 1983; Outridge and Hutchinson, 1990). Lemna

trisulca is a clonal plant which spreads and occupies space through the production of ramets, each of which consists of a single frond and root of determinate size. The branching pattern is dichotomous (Harper, 1985), with two main branch daughters being produced per parent frond, and occurs in three dimensions instead of the normal two (Cook, 1985). L. trisulca possesses persistent connections between ramets, grows in a relatively stable environment and spreads in a dense mat, all features of the phalanx growth form (Harper, 1985; Pitelka and Athmun, 1985). Outridge and Hutchinson (1990) have stated that exposure to Cd in Salvinia molesta increased allocation of resources to apical daughters at the expense of laterals. This would lead to earlier fragmentation and greater dispersal of plant material which is characteristic of the guerilla strategy (Harper, 1985).

The purpose of the first three experiments in this study was to explore, using Cd as a toxicant, three of the factors which could affect the precision and environmental relevance of toxicity data obtained using L. trisulca or other duckweeds. Experiment 1 examined the speed at which Cd was absorbed by L. trisulca and the lag period before the plant responded with a decrease in growth. Experiment 2 was designed to determine if tolerance to Cd could be induced in L. trisulca by several weeks exposure to sub-lethal Cd levels. Experiment 3 monitored the growth rate of L. trisulca over an extended period of time to determine if the effect of Cd on growth rate was dependent on fluctuations in the growth rate of control cultures. The purpose of the fourth experiment was to characterise clonal growth in L. trisulca and to examine the effect of Cd on the pattern of growth.

METHODS

Culture Methods

Axenic stock cultures of L. trisulca were maintained under standard conditions (Chapter 4). The basal medium (Table 4.1) had an initial pH of 7.8. For experiments,

either one or three fronds of L. trisulca were placed into 1-L Erlenmeyer flasks that contained 750 ml of filter-sterilized medium and were connected to a 4-L reservoir with 3250 ml of the same medium. A portion of the culture medium was replaced regularly by medium from the reservoir, doubling in amount approximately every 2 days. The temperature in the culture flasks was 28 ± 2°C. Flasks were illuminated with continuous cool-white flourescent light at 400 µmol/m^2/s PAR. Cultures were aerated at approximately 125 ml/min with ambient air that had been passed through a 0.45 µ sterile filter. After 14 days growth, plants in all experiments were removed from the culture flasks and fronds with roots were counted. Multiplication rate (MR) was calculated as;

$$MR = 1000 (\log F_t - \log F_o)/t$$

where F_t is the final number of fronds, F_o is the initial frond number, and t is the time in days (Hillman, 1961; Landolt and Kandeler, 1987). The doubling time was calculated as 301/MR (Landolt and Kandeler, 1987). Plants were oven dried, weighed, digested and analyzed for Cd as described in Chapter 5. The data from this chapter are contained in Tables A.6.1, 2, 3, 4, 5 in Appendix A. This chapter was published as Huebert and Shay (1992c).

The lag between exposure and toxicity (Exp. 1)

The first experiment examined how fast the internal Cd content of L. trisulca increased to toxic levels when the plant was exposed to Cd and the number of days before growth was reduced. In this experiment, basal medium lacking Cd was added to 24 flasks. On day 0, each flask was inoculated with a single frond of L. trisulca and the plants were grown for a total of 14 days. On day 0, three flasks were randomly chosen and Cd was added to both reservoir and culture flasks to increase the Cd concentration to 0.64 µM. This level of Cd decreases the MR approximately 50% (Huebert and Shay, 1991b). Every 2 days thereafter, Cd was added to another three, randomly chosen flasks. This resulted in eight treatments, all of which were cultured a total of 14 days, but

exposed to 0.64 μM Cd for 0, 2, 4, 6, 8, 10, 12, or 14 days. The three flasks with no added Cd were the controls. Growth was monitored by the change in the number of fronds, which were counted on day 0, 2, 4, 6, 8, 10, 12 and 14. Unfortunately, the number of fronds increased so rapidly in this experiment that fronds could not be counted on days 10 and 12 for treatments which were exposed to Cd for 8 days or less. Significant differences in frond number were tested according to Tukey et al. (1985).

Induction of Cd tolerance (Exp. 2)

The second experiment was designed to examine the induction of Cd tolerance in L. trisulca and rate of the recovery of L. trisulca after exposure to Cd. In this experiment, stock cultures of L. trisulca were pretreated in basal medium which was augmented with 0, 0.08, or 0.32 μM Cd. Every week for 6 weeks, or approximately 20 generations, plants in all three pretreatments were subcultured into fresh medium which also contained 0, 0.08, or 0.32 μM Cd. Plants were exposed to the same Cd level for the entire 6 weeks. To start the experiment, flasks which contained a basal medium plus 0, 0.16, 0.32 or 0.64 μM Cd were inoculated with a single frond from one of the three pretreatments. At the start of the experiment, L. trisulca plants pretreated with 0.08 μM Cd contained 87 ± 20 μg Cd/g dry wt. and those pretreated with 0.32 μM Cd contained 250 ± 94 μg Cd/g dry wt. The latter Cd content is high enough to reduce growth since the threshold for toxicity based on internal Cd is between 120 and 150 μg Cd/g dry wt. (Chapter 4). The actual external Cd concentrations were measured on the last day of the experiment and averaged 108% of the target concentration with a precision of 17% (as coefficient of variation). There were thus a total of twelve treatment combinations in a 4 by 3 factorial treatment structure in a split-plot design, with three replicates per treatment. Data were analyzed using two-way ANOVA. The assumption was made that variability in growth and interactions introduced by different flasks within a pretreatment was minimal. This assumption is reasonable because a single clone was used and the MR varied less than 3%

(as coefficient of variation) between flasks which contained the same medium (see Exp. 3 results). The assumption liberalized the decision to reject the null hypothesis in the ANOVA which increased the probability of a Type I error.

The effect of intrinsic multiplication rate on Cd toxicity (Exp. 3)

The third experiment was designed to monitor long-term changes in the intrinsic growth rate of L. trisulca and its response to 0.32 μM Cd. For Experiment 3, flasks containing either basal medium or medium plus 0.32 μM Cd (measured as 0.35 ± 0.06 μM Cd) were inoculated with either one or three fronds of L. trisulca. For each measurement of MR, the plants were grown for 14 days and harvested according to procedures discussed above. This was done 67 separate times under control conditions in basal medium and 33 times in basal medium augmented with 0.32 μM Cd. The experiment was started on Oct. 24, 1989 and spanned a period of almost 600 days.

The effect of Cd on growth patterns (Exp. 4)

The fourth experiment was designed to examine whether L. trisulca changed its branching pattern in response to Cd. For Experiment 4, plants were grown in control conditions and at 0.32 μM Cd. Fronds were counted daily and the branching pattern was recorded.

RESULTS

The lag between exposure and toxicity (Exp. 1)

In the control treatment without Cd, the relationship between $\log F_t$ and t was linear ($R^2 = 0.99$), which indicated that growth was exponential over the 14 day culture period (Fig. 7.1). The MR was 201 in the control, a doubling time of 1.5 days (Fig. 7.1). The number of fronds and hence the MR were significantly reduced 2 or 4 days after the addition of 0.64 μM Cd (Table 7.1) which suggests that the lag period was 2 ± 1 days.

Only treatments exposed to Cd for 10, 12 or 14 days are shown in Table 7.1 because fronds were too abundant to be counted accurately after 8 days growth in treatments with shorter Cd exposures. In treatments exposed to Cd for 10, 12 or 14 days, the MR was calculated on frond production from 2 days after Cd addition to the end of the experiment. Since $\log F_t$ vs t was linear ($R^2 = 0.90$ to 0.92; see Table 7.1, Fig. 7.1), MR was constant at the lower rate after the lag period. In the treatment in which plants were exposed to 0.64 μM Cd for 14 days, the MR was calculated from day 2 to day 14 of the experiment (Table 7.1). On this basis, 0.64 μM Cd reduced the MR by 68 ± 6%. If the MR had been measured over the entire 14 days without taking the lag period into account, then the reduction in MR caused by Cd would have been calculated at 56 ± 4%. The effect of a toxicant is therefore underestimated if the lag period is not removed from growth rate calculations.

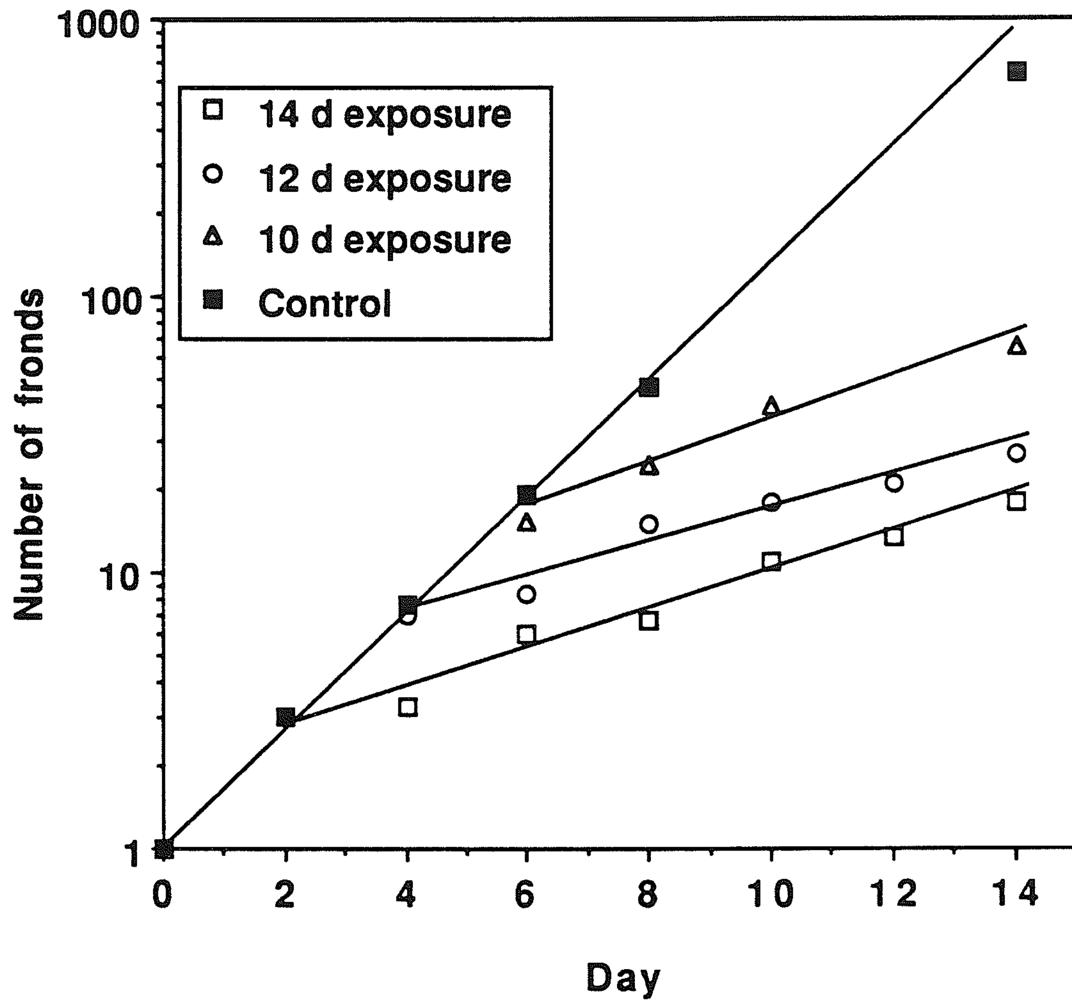
The final yield after 14 days culture was a linear function ($R^2 = 0.95$) of the number of days L. trisulca was exposed to 0.64 μM Cd (Fig. 7.2). Approximately 8% biomass was lost each day of exposure to 0.64 μM Cd (Fig. 7.2). The lowest biomass recorded was 6.8 ± 2.8 mg dry wt. in cultures exposed to Cd for 14 days. This was a 96% reduction in biomass compared with the control treatment, where the final yield biomass was 178 ± 20 mg dry wt. (Fig. 7.2).

In the treatments where Cd was added on day 12 and the plants were exposed to Cd for only 2 days, the Cd content of L. trisulca was 1,000 ± 140 μg Cd/g dry wt (Fig. 7.3). Cadmium content increased to 3,800 ± 200 μg Cd/g dry wt. at longer exposure times (Fig. 7.3). This is misleading, however, because the external Cd concentration was reduced at shorter exposure times (Table 7.2), which probably also lowered the internal Cd content. The external Cd concentration was not maintained because as the length of exposure to Cd was reduced there was a linear increase in the final yield (Fig. 7.2) and a corresponding linear increase in the percent of the total amount of Cd contained within the plant (Table 7.2). For instance, the μg of Cd contained within the plant after 2 days

Table 7.1. The response of Lemna trisulca to the addition of 0.64 μM Cd in Experiment 1. Treatments, as number of days exposure to 0.64 μM Cd, are shown on the far left. The control had no Cd added. Treatments were tested for a significant (NS = not significant, * = significant at $p < 0.01$) reduction in frond number on the day that Cd was added and two and four days later. The correlation coefficient (R^2) and multiplication rates (MR) were calculated from the linear regression, (log F_t vs t), from 2 days after the addition of Cd to the end of the experiment, which lasted 14 days. For all treatments, data are presented as mean ± sd, n = 3.

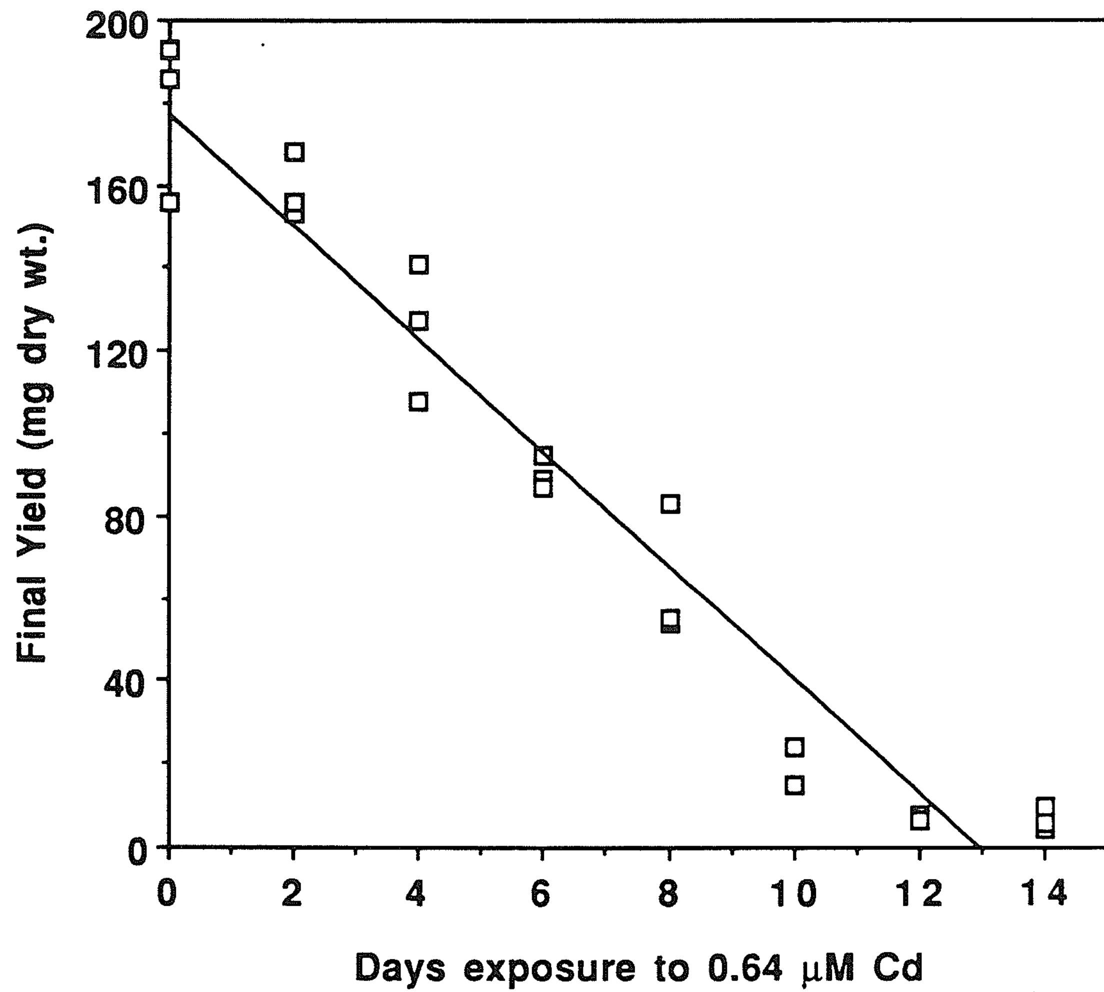
Number of days	Days after addition of Cd			R^2	MR
exposure to Cd	0	2	4		
Control	___	___	___	0.99	201 ± 4
10 d	NS	*	*	0.91	79 ± 8
12 d	NS	NS	*	0.90	58 ± 4
14 d	NS	NS	*	0.92	64 ± 10

Figure 7.1. The effect of 0.64 μM Cd on frond production in Lemna trisulca in Experiment 1. All treatments were inoculated with plants at day 0; Cd was then added to three randomly chosen flasks at 2 days intervals starting on day 0. Data are shown for only 10 (open triangle), 12 (open circle) and 14 days (open square) exposure. For control treatments lacking Cd (closed square), y = 0.20x - 0.16, R^2 = 0.995. Means of three replicates per treatment are shown.



14 d exposure
12 d exposure
10 d exposure
Control
Number of fronds
1000
100
10
1
0
2
4
6
8
10
12
14
Day

Figure 7.2. Final yield of Lemna trisulca versus the number of days exposure to 0.64 µM Cd in Experiment 1, where y = -13.7x + 177, R^2 = 0.95. All cultures were grown for a total of 14 days and there were three replicates per treatment.



Final Yield (mg dry wt.)
200
160
120
80
40
0
0
2
4
6
8
10
12
14
Days exposure to 0.64 μM Cd

exposure represented 90% of the total μg of Cd available for uptake (Table 7.2). Plants exposed to Cd for 14 days, on the other hand, contained less than 10% of the total available Cd and the external Cd remained at the nominal concentration (Table 7.2).

Induction of Cd tolerance (Exp. 2)

The MR decreased an average of 13% at 0.16 μM Cd, 34% at 0.32 μM Cd and 60% at 0.64 μM Cd (Fig. 7.4). Pretreating L. trisulca with 0.08 or 0.32 μM Cd had no significant effect on MR ($p = 0.05$, $F = 0.71$, 2 df) when plants were exposed to a Cd challenge of 0.16, 0.32 or 0.64 μM Cd. Cadmium tolerance was therefore not induced in L. trisulca when MR was used to measure Cd toxicity. There was also no significant interaction between pretreatment and the subsequent MR ($p > 0.05$, $F = 0.86$, 6 df) for L. trisulca. Plants grown in basal medium lacking Cd had the same MR whether or not they were pretreated in a medium containing Cd (Fig. 7.4).

The internal Cd content averaged less than 2 μg/g dry wt. in treatments lacking Cd, 190 ± 45 μg Cd/g dry wt. in 0.16 μM Cd, 570 ± 130 μg Cd/g dry wt. in 0.32 μM Cd and 1,800 ± 500 μg Cd/d dry wt in 0.64 μM Cd (Fig. 7.5). There was no significant effect of pretreatment ($p > 0.05$, $F = 0.06$, 2 df) or interaction between pretreatment and external concentration ($p > 0.05$, $F = 0.20$, 6 df) on Cd uptake (Fig. 7.5).

The effect of intrinsic multiplication rate on Cd toxicity (Exp. 3)

The MR of L. trisulca was measured repeatedly in 14 day time periods under standard conditions. Almost 600 days elapsed between the first and last measurement. During this time, the stock cultures used to inoculate the experimental cultures were maintained under constant light, temperature and nutrient conditions. The MR periodically increased and decreased (Fig. 7.6). The MR for single observations varied between a low of 125, which is a 2.4 day doubling time, and a high of 183, which is a 1.6 day doubling time (Fig. 7.6). After 14 days growth, there was greater than a five-fold difference in the

Table 7.2. The effect of number of days exposure to 0.64 µM Cd on the external Cd concentration (µM) and the percent uptake in Lemna trisulca in Experiment 1.

	Days exposure to 0.64 µM Cd						
Parameter	2	4	6	8	10	12	14
Cd[a]	0.35 ± 0.03	0.23 ± 0.02	0.34 ± 0.04	0.42 ± 0.02	0.64 ± 0.09	0.64 ± 0.01	0.64 ± 0.02
% uptake[b]	90 ± 16	45 ± 12	53 ± 13	42 ± 10	19 ± 1	10 ± 1	6 ± 1

a. Measured in the culture flask on day 14.

b. Percent uptake was measured as the total amount of Cd (in µg) contained within L. trisulca at the end of the experiment, divided by the total amount of Cd which was added to the experimental unit .

Figure 7.3. The internal Cd content (μg Cd/g dry wt.) of Lemna trisulca versus the number of days exposure to 0.64 μM Cd in Experiment 1. Data are presented as the mean ± sd, n = 3.

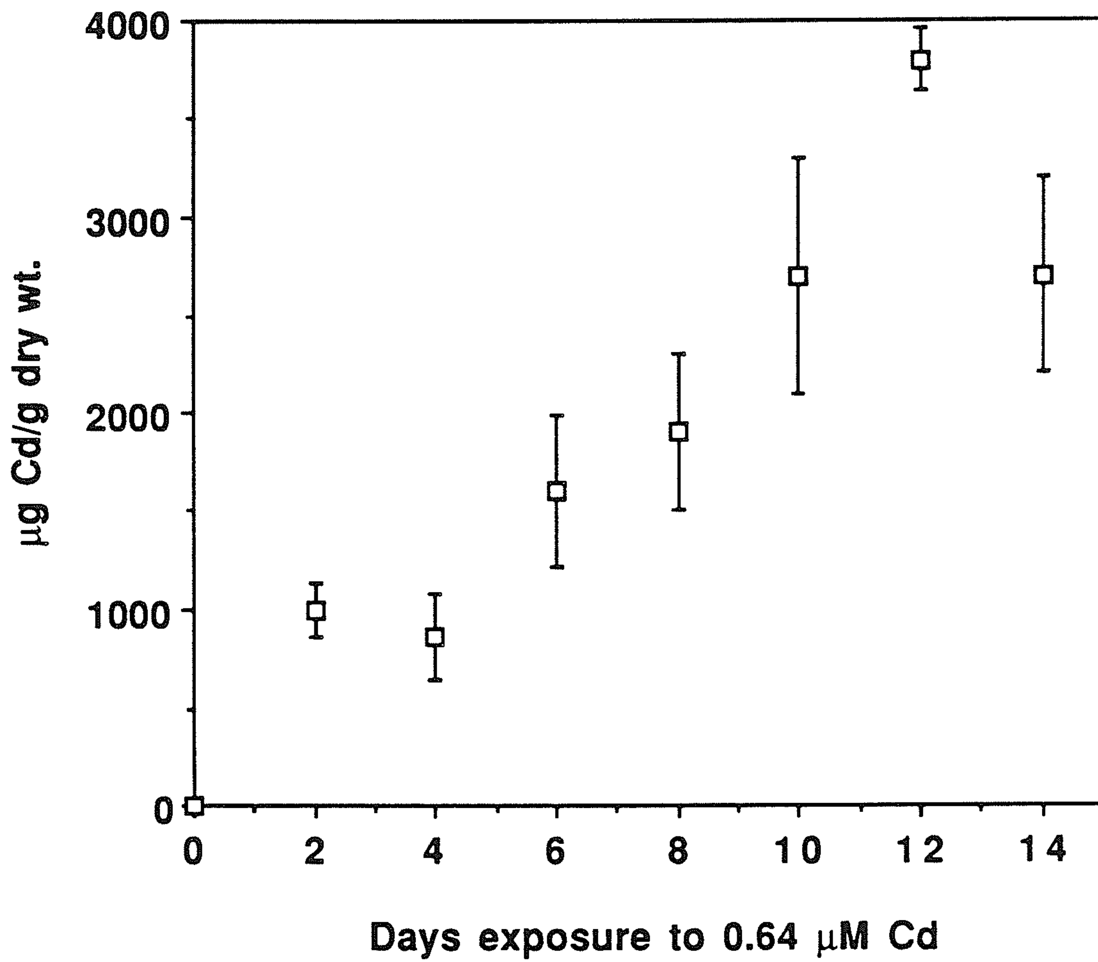
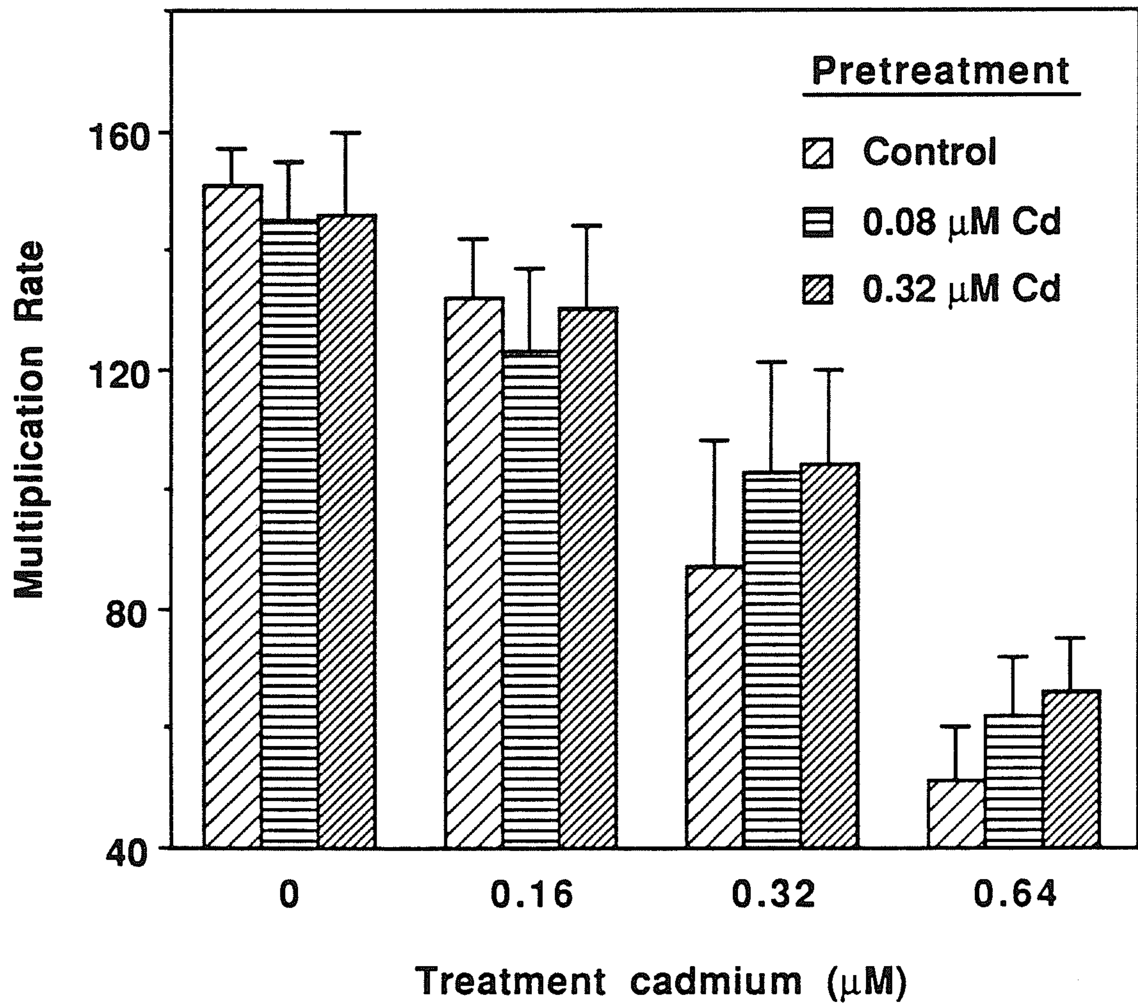

4000
3000
2000
1000
0
μg Cd/g dry wt.
0
2
4
6
8
10
12
14
Days exposure to 0.64 μM Cd

Figure 7.4. The effect of pretreatment on Cd toxicity in Lemna trisulca in Experiment 2. Plants were pretreated for 6 weeks under control conditions, or with 0.08 or 0.32 μM Cd. The abcissa represents the four Cd treatments and the bars are the three pretreatments. Data are presented as the mean ± sd, n = 3.



Pretreatment
Control
0.08 μM Cd
0.32 μM Cd
Multiplication Rate
160
120
80
40
0
0.16
0.32
0.64
Treatment cadmium (μM)

Figure 7.5. The effect of pretreatment on Cd uptake (μg Cd/g dry wt.) in Lemna trisulca for Experiment 2. Plants were pretreated for 6 weeks under standard conditions, or with 0.08 or 0.32 μM Cd, and then exposed to four levels of Cd (which are shown along the abscissa).

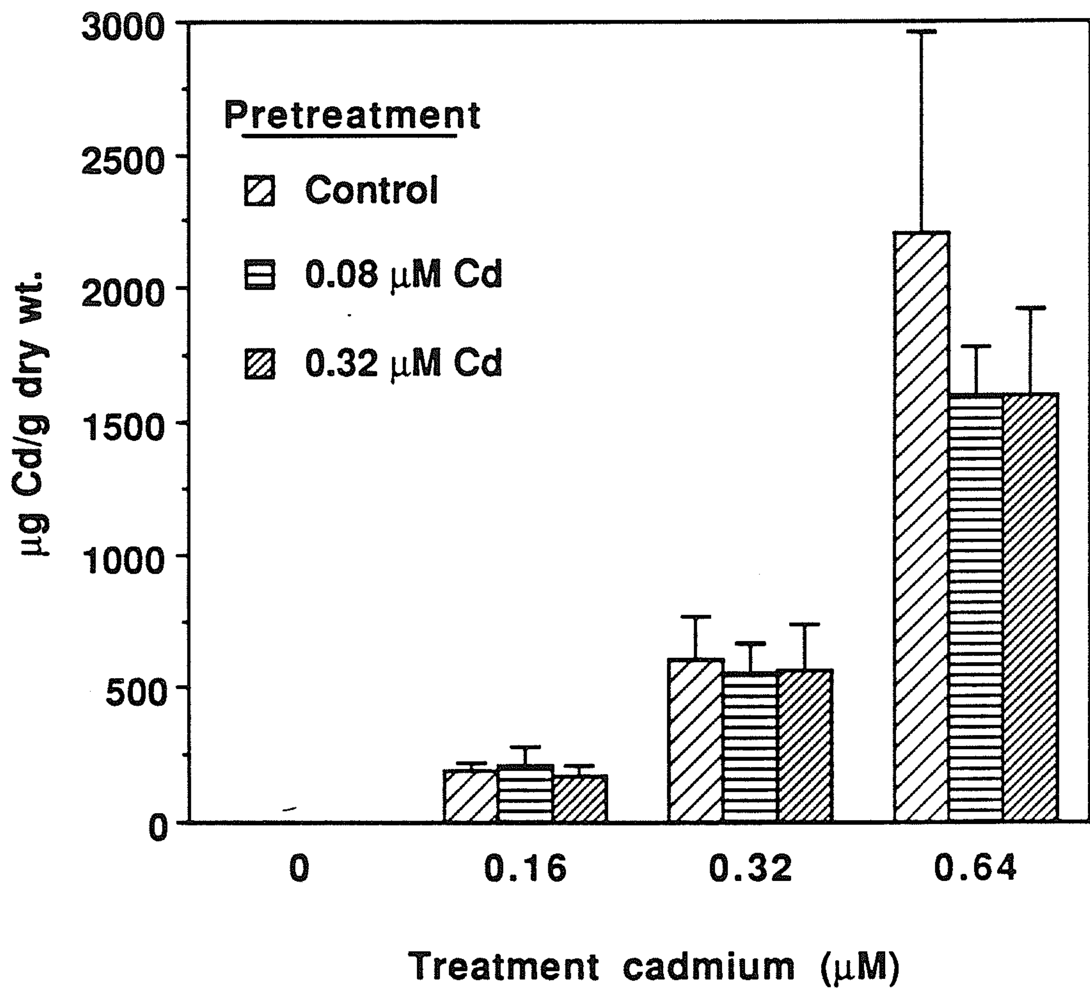
final yield between these two extremes. This is a large difference since replicates for a given date typically varied less than 3% in MR (as coefficient of variation). The frond biomass did not appear to change over this same time period since the mean ± sd of frond dry wt. of all samples was 260 ± 24 µg/frond. This was similar to the variability found for replicates (data not shown).

The effect of 0.32 µM Cd on the MR of L. trisulca was also measured repeatedly over approximately 600 days and varied with the MR of control treatments (Fig. 7.7), though the relationship was weak ($R^2 = 0.44$). Cadmium at 0.32 µM Cd reduced the MR an average of 24%, with a coefficient of variation of 38%. The median reduction in MR caused by 0.32 µM Cd was 22% and the data was slightly skewed upwards, with three outliers above 40% (boxplot, Fig. 7.7).

The effect of Cd on growth patterns (Exp. 4)

The original parent frond was still viable after 14 days growth in control cultures and at 0.32 µM Cd. Both the left and right daughter fronds developed synchronously. Growth of daughter fronds was perpendicular to the long axis of the parent frond and at 0° in the horizontal plane. Petioles were persistent in both treatments, no degradation being observed after 14 days growth through 5 (0.32 µM Cd) or 6 (control) generations. The plants spread in three dimensions by a 90° twist in the petiole as it developed; one daughter frond twisting forward and the other back. Three frond fragments had blades oriented in all three dimensions.

New main-branch daughter fronds were produced every 1 to 3 days (mean 2.2, n = 70) in control cultures and every 1 to 4 days (mean 3.0, n = 59) at 0.32 µM Cd (Table 7.3). In both treatments, main-branch daughters of one generation were produced synchronously, with an entire generation appearing within approximately 24 h. Some time after the main-branch daughters were produced, side-branches began to develop.



3000
2500
2000
1500
1000
500
0
μg Cd/g dry wt.
Pretreatment
Control
0.08 μM Cd
0.32 μM Cd
0
0.16
0.32
0.64
Treatment cadmium (μM)

Figure 7.6. The multiplication rate (MR) of Lemna trisulca measured repeatedly under standard conditions over a period of almost 600 days in Experiment 3. The line was drawn through the mean of the data using a Spineman interpolation (Spineman, 1980).

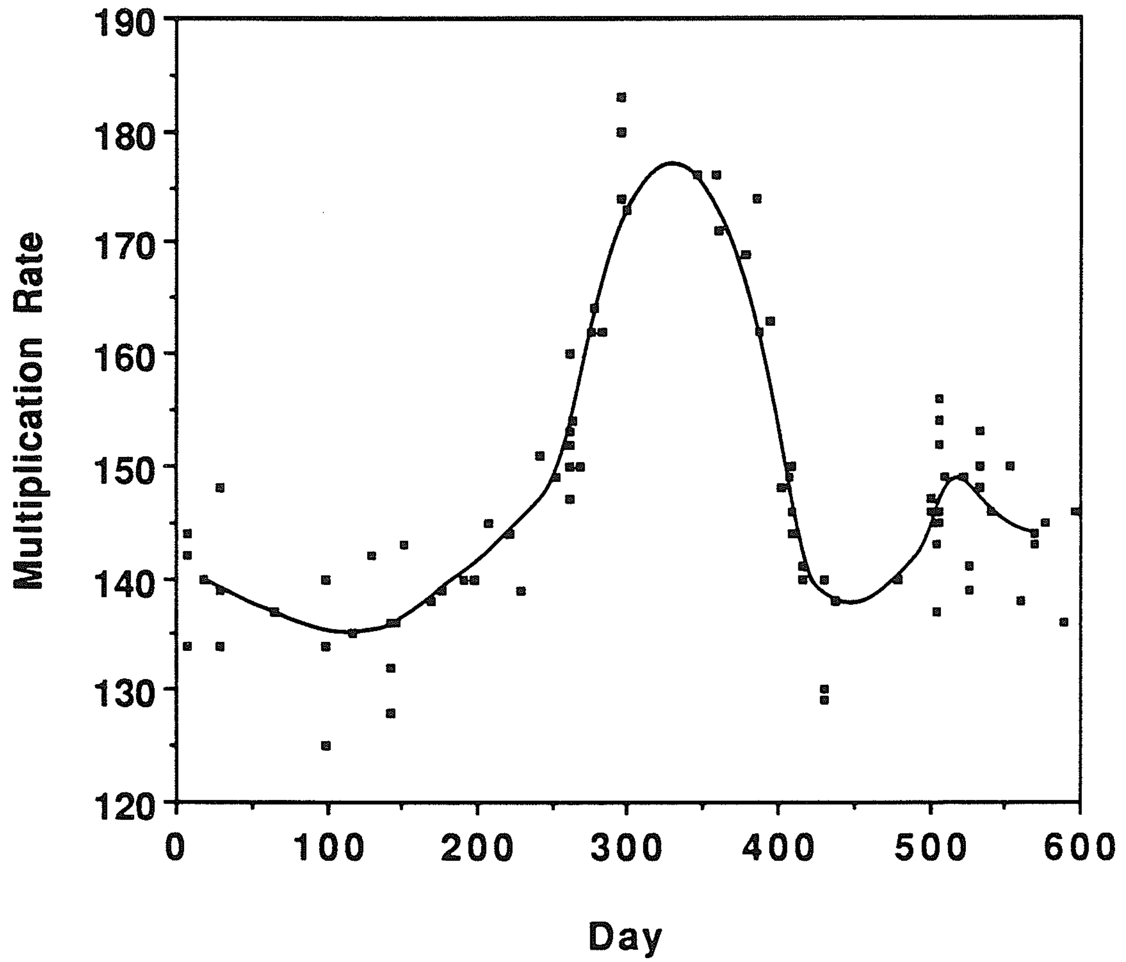
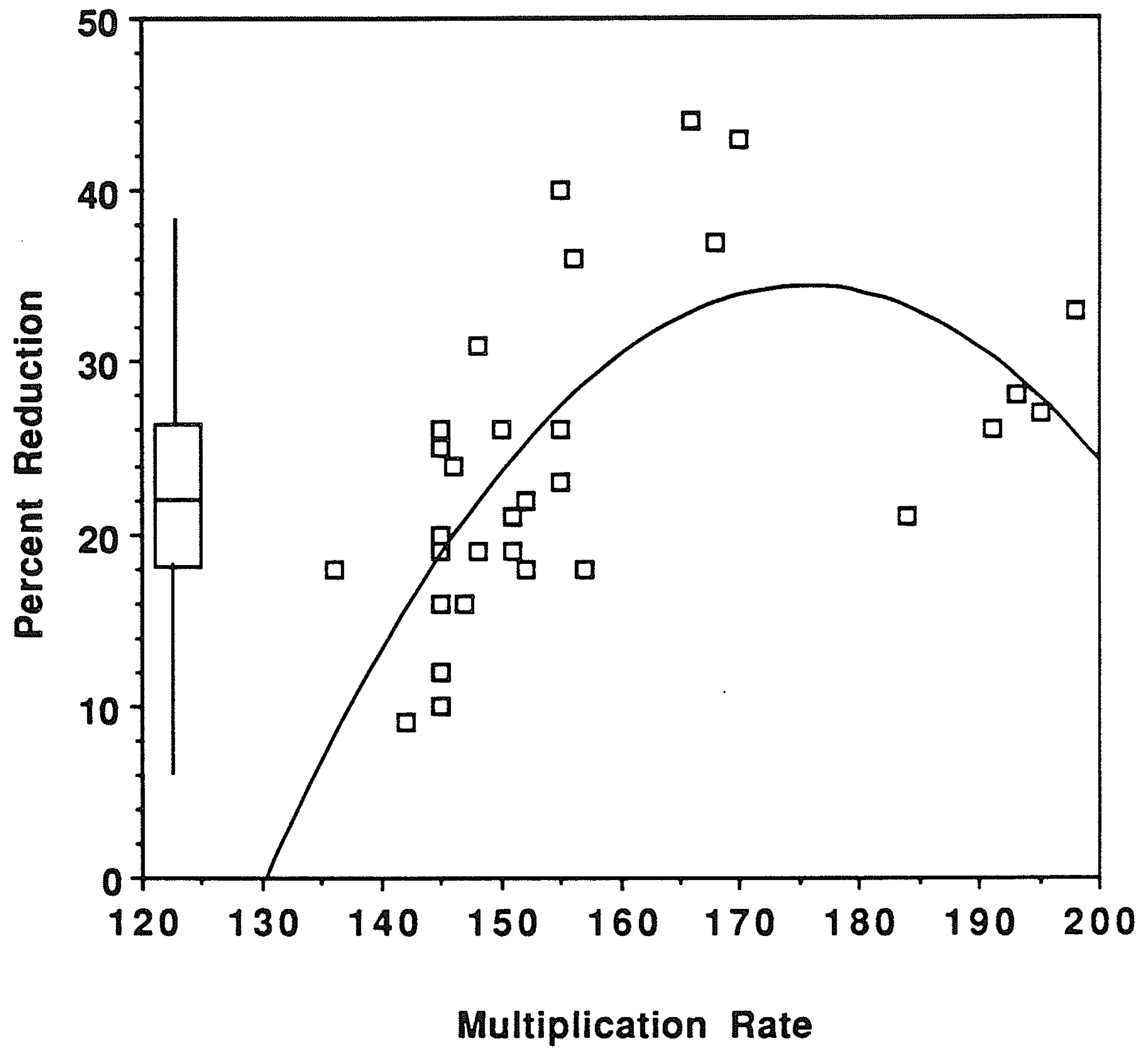

190
180
170
160
150
140
130
120
Multiplication Rate
0
100
200
300
400
500
600
Day

Figure 7.7. The relationship between multiplication rate (MR) in Lemna trisulca and percent reduction in MR caused by 0.32 μM Cd in Experiment 3; $y = 5.9x - 0.02 x^2 - 490$, $R^2 = 0.44$. The boxplot indicates the median ± one quartile of data. The lines above and below the boxplot are a maximum of 1.5 times the interquartile range. Values beyond these lines are designated as outliers.



Percent Reduction
Multiplication Rate
0
10
20
30
40
50
120
130
140
150
160
170
180
190
200

For the original parent frond, first generation side-branch daughters were produced on day 6 and days 12 to 14 in the control and on day 13 at 0.32 μM Cd (Table 7.3). In control cultures, side-branches developed, on average, 6.4 days after the parent frond. Amongst parent fronds of the same generation there was much less synchronicity in the development of side-branches, with a range in development of from 4 to 10 days. In addition, only one side-branch developed at a time. In the control treatment , the original parent frond produced three side-branches in 14 days and all fronds existing for more than 7 days had at least one side-branch (Table 7.3). In contrast, side-branch development at 0.32 μM Cd was minimal (Table 7.3).

Because of the synchronicity of daughter frond production in each generation, every modular unit was symmetrical with others containing the same number of generations. Modular units were of size 3, 7, 15, 31, 63 or 127 for the six generations produced in the control cultures. Differences in modular size could be ascribed entirely to variability in the production and growth of side branches.

Plants grown at 0.32 μM Cd produced five generations after 14 days growth, only one generation less than control cultures (Table 7.3). In control cultures, however, 76 side-branch fronds were produced compared to only 3 in plants grown at 0.32 μM Cd. Total frond production was 203 (MR = 165) in control cultures and 63 (MR = 128) at 0.32 μM Cd. Based on MR, the presence of 0.32 μM Cd inhibited growth by 22.5%, which is close to the average reported earlier. Final yield was inhibited by 53% based on daughter frond production and by 96% based on side-branch production.

DISCUSSION

The lag between Cd exposure and toxicity (Exp. 1)

When L. trisulca was exposed to 0.64 μM Cd, there was a lag period of about 2 days before the MR was reduced. The lower MR was then maintained for

Table 7.3. Frond production in Lemna trisulca under control conditions and 0.32 μM Cd.

	Day														
Generation	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
							Control								
P	1	1	1	1	1	1	1	1	1	1	1	1			1
1		2	2	2	2	2	3	3	3	3	3	3			5
2					4	4	4	4	4	7	7	8			10
3							5	8	8	9	12	17			23
4									2	16	16	20			46
5											4	32			54
6															64
SB[a]							1	1	1	5	8	18			76
Σ	1	3	3	3	7	7	13	16	18	36	43	81			203[b]
							0.32 μM Cd								
P	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1		2	2	2	2	2	2	2	2	2	2	2	2	3	3
2					4	4	4	4	4	4	4	4	4	4	6
3									8	8	8	8	8	8	8
4											1	16	16	16	16
5														5	29
SB[a]														1	3
Σ	1	3	3	3	7	7	7	7	15	15	16	31	31	37	63[b]

a. SB indicates the number of side-branch fronds as distinct from main-branch daughters.

b. The multiplication rate equals 165 for 203 fronds and 128 for 63 fronds.

the remainder of the experiment, as seen by the linear relationship between $\log F_t$ versus t and between length of exposure to Cd versus final yield. The rapid and constant response of L. trisulca to Cd suggests that, at least under these conditions, it is not necessary to culture the plant for four weeks under constant conditions before growth rates are measured, (as in Landolt and Kandeler, 1987). It is essential, however, that the lag period be determined for each toxicant investigated, since this parameter may very well change for different toxicants. This study did not determine whether the lag period changed at different Cd concentrations, although observations suggested that the lag period was constant over a wide range of Cd.

When the plants were exposed to 0.64 µM Cd for 14 days, the MR was reduced by 68% when calculated on frond production between day 2 and day 14, but only by 56% when the lag period was ignored and the MR was calculated from day 0 to day 14. If the lag period is not considered in the calculation of growth rate, the effect of a toxicant will be underestimated. As the length of the toxicity test is reduced, this underestimation will increase, since the higher growth rate that occurs in the lag period will become a larger fraction of the final yield. This further suggests that the underestimation of toxicity will also increase as the difference between the growth rate of the control and the growth rate in the presence of a toxicant increases. Results would, of course, be completely confounded if the lag period was greater than the duration of the toxicity test.

Lemna trisulca contained 1,000 ± 140 µg Cd/g dry wt. after 2 days exposure to 0.64 µM Cd. Plants exposed to Cd for 10 to 14 days contained up to 3,800 ± 200 µg Cd/g dry wt. These data are similar to those of Kwan and Smith (1991) who found that the internal Cd content of L. minor increased sigmoidally over time and reached a 'steady state' after approximately 300 h. From the data presented here, however, it is difficult to judge whether the plants were at 'steady state' with the external concentration at any given time. Firstly, plants exposed to Cd for 10 to 14 days had unexpectedly high Cd contents. Results from Chapter 4 suggested that the Cd content saturated in L. trisulca at between

1,500 to 3,000 μg Cd/g dry wt. at external concentrations as high as 1.28 μM. Secondly, as the length of exposure to Cd was reduced, biomass increased and sequestered an increasing amount of Cd which caused a reduction in the external Cd concentration. In fact, data from Chapter 4 shoe that the average Cd content ranged from 1,400 to 2,200 μg Cd/g dry wt. at 0.64 μM Cd. This is only slightly higher than the value reported here for the 2 days exposure treatment. It is certain, however, that a toxic amount of Cd had been absorbed by L. trisulca after 2 days exposure to 0.64 μM Cd, since the threshold for toxicity of internal Cd in L. trisulca is in the range of 120 to 150 μg Cd/g dry wt.

The efficiency with which L. trisulca absorbed Cd suggests that static culture techniques (Wang, 1990; ASTM, 1991; Wang, 1991) are inadequate when assessing metal toxicity using duckweeds. This is of particular concern in studies where it is unlikely that the nominal metal concentration would be maintained over the test period because of a large inoculum and low volume of medium. If the actual concentration of metal was not reported, the reduction in metal levels during the experiment could compromise the measurement of toxicity.

Induction of Cd tolerance (Exp. 2)

It has been known for some time that metal tolerant plants exist and that these species grow in contaminated environments (Antonovics, 1971; Baker, 1987). Possible mechanisms of metal tolerance have also been discussed (Trevors et al., 1986; Taylor et al., 1987). Presumably, in cases of metal tolerance, an induction of tolerance occurred when the plants were first exposed to elevated metal concentrations. In my experiment, however, pretreating L. trisulca for 6 weeks with 0.08 or 0.32 μM Cd had no significant effect on, or interaction with, the MR and uptake of Cd when the plants were subsequently exposed to various Cd concentrations. There was no indication in the data that Cd tolerance was induced even after 6 weeks of exposure to Cd, despite preliminary observations which suggested that L. trisulca altered its growth after lengthy exposure to

sub-lethal Cd concentrations. Plants pretreated with toxic levels of Cd also showed no significant reduction in MR when subsequently placed into basal medium lacking Cd. This suggests that L. trisulca recovers quickly from Cd exposure.

Outridge et al. (1991) and Outridge and Hutchinson (1991), in a similar experiment, examined the effect of 15 or 25 ppb Cd on Salvinia minima plants pretreated with 0, 10, 25, or 50 ppb Cd. While there was clear evidence that exposure to 25 ppb Cd increased phytochelatins (PC) in the plants (Outridge et al., 1991), there was no statistical evidence to suggest that pretreatment altered the amount of PC produced when the plants were subsequently exposed to 25 ppb Cd. There were also few consistent effects of pretreatment on biomass, chlorophyll a, metal binding proteins or thiols. Their data suggest that changes occur when S. minima is exposed to Cd (as evidenced by the increase in PC) but the results are not consistent enough to provide a definitive answer as to whether these changes increase the tolerance of the plant to Cd. Since the data in these two papers were analyzed using a series of one-way ANOVA's, it is impossible to tell whether a pretreatment by Cd interaction could have contributed to their inconsistent results.

My results and those of Outridge et al. (1991) and Outridge and Hutchinson (1991) suggest that more work needs to be done to characterize the long-term response of aquatic plants to Cd. It is important to examine this question since aquatic plants are increasingly used to assess aquatic toxicity. If tolerance can be induced, then the relevance of toxicity data could be compromised by changes in the effect of a toxicant depending on the length of exposure to that toxicant.

The effect of intrinsic multiplication rate on Cd toxicity (Exp. 3)

There was an increase followed by a decrease and then another increase in the intrinsic growth rate of L. trisulca over a period of almost 600 days. The final yield in 14 day cultures differed more than five-fold between the highest and lowest measured MR.

These differences are large considering that the MR varied less than 3% (as coefficient of variation) amongst replicates. The fluctuations in MR occurred despite constant, rigorously controlled conditions both in the stock cultures and experimental flasks. These results are consistent with other studies which have also found that the growth rates of aquatic macrophytes vary throughout the year (Huebert and Gorham, 1983; Landolt and Kandeler, 1987; Wang, 1990). The attempt to control the fluctuations in intrinsic growth rate (Chapter 2) by cycling the plants through a dormancy period was therefore a failure.

The variability in intrinsic growth rate over time is of concern when the effect of a toxicant on growth rate is being examined, since the data are compared with a control value. Changes in the growth rate of the control could mask any toxicant effect and compromise the assessment of toxicity. To avoid this problem, I recommend, as do Landolt and Kandeler (1987), that complete experiments be carried out simultaneously. If this is not possible because of space or equipment constraints, then a complete block design should be used where replicates are blocked over time and any variability in growth rate over time can then be separated by ANOVA as a block effect.

Potentially more serious is that the response of L. trisulca to Cd appeared to vary with the intrinsic growth rate ($R^2 = 0.44$), which suggests that the effect of a toxicant may be governed by undefined variation in the growth potential of the test plant. In contrast, Wang (1990) found no cyclic changes in the effect of the reference toxicant Cr to L. minor, though he did not examine the relationship between growth rate and the effect of a toxicant. In my study, however, this relationship was weak and perhaps could be incorporated within the variability of the test procedure.

When any time dependent relationships were ignored, the median response of L. trisulca to 0.32 µM Cd was a 22% reduction in MR with half the data between 18 and 26%. The mean response was a 24% reduction and the coefficient of variation was 38%. This is in the range of variability found for other bioassay procedures (Chapman, 1991; Anderson and Norberg-King, 1991). Even though the variability in the data in my study

was within limits found elsewhere, the potential for confounding the measurement of toxicity by changes in the intrinsic growth rate of the test plant, or by the plants response to a toxicant, must be recognized and considered. Wang (1990), for instance, suggests monitoring with both a negative and a positive control to ensure the quality of test results.

The effect of Cd on growth patterns (Exp. 4)

Lemna trisulca is a rather unique clonal plant in that it spreads in three dimensions, unlike most other clonal plants which spread in two dimensions over a soil/air, soil/water or water/air interface (Cook, 1985). Lemna trisulca spreads in three dimensions by a twist in developing petioles which allows fronds to orient themselves in all directions. Under severely stressed conditions this twist may not occur, in which case L. trisulca becomes planar and similar to other members of the Lemnaceae (Landolt, 1986). Another interesting feature of the general growth of L. trisulca was the synchronicity observed in the emergence of complete generations of daughter fronds. This suggests some form of developmental integration amongst ramets.

The number of generations produced by L. trisulca in 14 days was reduced by only one in the presence of 0.32 µM Cd. The number of side-branch fronds, however, decreased by 96%. The data suggest that L. trisulca reallocates resources to apical development when exposed to a stress such as Cd. This is in agreement with the work of Outridge and Hutchinson (1990) who found that Salvinia molesta increased apical growth at the expense of lateral development in the presence of 0.90 µM Cd. It is possible that this reallocation of resources to the apical meristem may represent a change from phalanx to a guerilla growth strategy (Harper, 1985). Phalanx growth is characterized by very dense packing with relatively short rhizomes which has the effect of consolidating a position in space. This strategy is likely to be found in a uniform, competitive environment. In contrast, a guerilla growth strategy is characterized by loose packing and relatively long rhizomes, which has the effect of allowing for greater dispersal of ramets.

Increased dispersal would be advantageous to *L. trisulca* in escaping from polluted or nutrient poor sites.

Chapter 8. Summary

An appropriate nutrient medium and adequate environmental conditions are the basis for any experimental studies which use aquatic plants. The first objective of this study, therefore, was to determine the conditions necessary for the successful and consistent culture of L. trisulca. To this end, L. trisulca was grown in axenic cultures at pH 7.8 in a medium without glucose or vitamins at a relatively high light intensity of 400 $\mu mol/m^2/s$ PAR. Growth was optimal when the cultures were aerated and a portion of the medium was replaced regularly. The volume of medium, replacement schedule and inoculum size were designed to ensure that the MR was constant and that growth was exponential through the 14 day test period. These conditions supported a multiplication rate as high as 200 (1.5 day doubling) and are radically different from those used for the past 30 years to grow L. trisulca.

Under the conditions developed in this study, the nutrient medium was optimized for P and Ca. The critical phosphate content for L. trisulca was 0.45 to 0.65 % dry wt. P, which occurred at an external concentration of 4.2 to 15 μM PO_4. Growth of L. trisulca was normal between 27.5 and 220 μM Ca. Within this range, the internal Ca content rose from 0.83 to 3.29% dry wt. Above 220 μM Ca, growth was inhibited, and at levels of Ca below 27.5 μM Ca, plants became small, twisted and deformed.

Unfortunately, even under these optimal and rigorously controlled conditions, the growth of L. trisulca was not constant over time. The MR ranged from a low of 125 to a high of 183 over a period of approximately 600 days. This produced greater than a five-fold difference in final yield in cultures grown for 14 days.

Interestingly, the effect of 0.32 μM Cd on MR varied with the MR of control treatments, though the amount of variability accounted for by this relationship was small ($R^2 = 0.44$). The median reduction in MR caused by 0.32 μM Cd was 22% with half the

data between 18 and 26% and was skewed upwards, with three outlies above 40%. The average reduction was 24% with a coefficient of variation of 38%.

For quality control of toxicity tests, the variability in growth of L. trisulca and the variability in its response to Cd must be monitored. This can be done by the inclusion in all toxicity experiments of both a negative control, which contains no toxicant, and positive control, which contains a known reference toxicant. If this is not done, results may be confounded by unknown changes in intrinsic growth rate and toxicant effect.

The second objective of this study was to examine the effect of chemical and biological factors on Cd uptake and toxicity. It is important to understand these interactions if toxicity data are to have any predictive value.

The toxicity of Cd to L. trisulca was determined as a basis for the examination of interactions between Cd and other elements. The Cd NOEC for L. trisulca was 0.08 μM external Cd for both MR and final yield, and between 116 and 147 μg Cd/g dry wt. internal Cd for MR. The Cd EC50 for L. trisulca was estimated at 0.99 μM external Cd for MR and 0.56 μM Cd for final yield. The precision of the measurement of toxicity based on MR was 38% as coefficient of variation. The discrepancy between the EC50 for MR and final yield appears to be due to external conditions. The toxicant concentration needed to produce a specific reduction in final yield depends not only on the reduction in MR, but also on the length of time the plant is cultured and on the MR of the control treatment. The faster the MR of control cultures and the longer the culture time, the lower the percent reduction in MR and the lower the toxicant concentration needed to produce a final yield EC50. This means that comparisons of toxicity based on final yield cannot be made between studies carried out in different laboratories or at different times.

Calcium was the first chemical factor examined. Surprisingly, there was no significant effect of Ca, or interaction between Ca and Cd, on MR, final yield, Cd content or frond weight in L. trisulca under the conditions of this study. These results are contrary to all other published data. The reason for this is unknown, but it raises the

question of whether the effects of Ca on Cd toxicity are due to the precipitation of Ca compounds and subsequent reduction in the Cd concentration, or to an actual antagonism of Cd toxicity.

Zinc was the second factor examined. The interaction between Cd and Zn is of interest because they are often found in association with each other. The Zn EC50 for L. trisulca was estimated at 16 μM external Zn for MR and 7.8 μM for final yield. There was a significant antagonistic interaction between Cd and Zn in L. trisulca. The multiple regression model developed from the MR data indicated that at low Zn concentrations the toxic effect of Cd was pronounced, but that increased Zn levels eliminated the Cd effect. The MR at a specific Cd/Zn combination could be largely predicted by the Zn concentration alone at higher Zn levels. The final yield in treatments containing Cd at elevated Zn levels averaged approximately 1.5 times those observed in cultures grown with Cd at control levels of Zn.

The internal Cd content was significantly affected by both the external Zn and Cd concentration, but there was no significant Cd by Zn interaction. Increasing the Zn concentration up to a level of Zn which was itself not toxic (3.06 μM) decreased Cd uptake. Further increases in the Zn concentration increased Cd uptake.

The data suggest that Zn, when it is at a much higher concentration than Cd both internally and externally, competitively excludes Cd from entering the cell at non-toxic Zn concentrations. Zinc then excludes Cd from internal binding sites as the internal Cd content increases at higher, toxic external Zn concentrations. This mitigates Cd toxicity at all Zn levels. Because of these interactions, metal toxicity studies should be carried out with a mixture of metals rather than with single metals in isolation.

The most dramatic interaction was between EDTA and Cd or Zn. When the amount of available EDTA exceeded the concentration of Cd or Zn, uptake was reduced by 95 to 99%. Excess available EDTA strongly antagonized the toxic effect of Cd and Zn on MR and final yield.

The amount of EDTA available for chelating Cd or Zn was determined as that amount in excess of the Fe concentration. As the Fe concentration was lowered, Cd uptake and toxicity decreased. These data suggest that the Fe/EDTA ratio is a critical component of any lab study on metal toxicity. Unfortunately, this is an intractable problem because of the complex aqueous chemistry of Fe.

The antagonistic effect of EDTA supports the hypothesis that in L. trisulca, as in other aquatic organisms, it is the free ion activity of Cd and Zn and not their total concentration which determines their uptake and toxicity. The implication is that for toxicity tests of metals, it is imperative that the EDTA concentration is defined initially and controlled throughout the test period. No confidence can be placed in metal toxicity tests if the chelating properties of the medium are undefined and uncontrolled.

Biological factors which potentially alter the toxic effect of Cd were also examined. For instance, the addition of 0.64 µM Cd to the culture medium significantly reduced the number of fronds after 2 ± 1 days compared with controls. The final yield after 14 days growth was a linear function of the number of days the plants were exposed to 0.64 µM Cd. The internal Cd content equilibrated after 2 days exposure to 0.64 µM Cd and saturated at 1,000 to 3,000 µg Cd/g dry wt. If the lag period is not removed from the calculation of MR, the effect of a toxicant will be underestimated. As the length of the toxicity test is reduced, this underestimation will increase since the higher MR that occurs in the lag period will constitute a larger and larger fraction of the final yield.

When L. trisulca was pretreated for 6 to 9 weeks with 0.08 or 0.32 µM Cd and then exposed to 0, 0.16, 0.32 or 0.64 µM Cd, there was no significant effect of pretreatment, or pretreatment by Cd interaction, on MR or Cd uptake. This suggests that L. trisulca does not become acclimated to the external Cd concentration and that induction of Cd tolerance did not occur.

New main branch daughter fronds were produced every 1 to 3 days in control cultures and every 1 to 4 days at 0.32 µM Cd. L. trisulca branches dichotomously and,

for the clone used here, both left and right daughter fronds developed synchronously. Main-branch daughters of one generation were also produced synchronously in the entire clone, with one generation appearing within approximately 24 h. Side-branches developed every 6.4 days after the appearance of the parent frond in control cultures. Side-branch development was reduced by 96% at 0.32 μM Cd. This suggests that L. trisulca reallocates resources to apical development at the expense of lateral spread when exposed to a stress such as Cd.

In conclusion, the response of L. trisulca to Cd is not a constant but is affected by many different biological and chemical factors. One of the most important factors is the chelating capacity of the medium. If this is unkown, toxicity cannot be predicted from the external Cd concentration alone. Furthermore, if chelation and other fators are not considered, such as in simplified bioassay protocols, toxicity studies on Cd and other metals will produce inconsistent results. This is dangerous, since it is laboratory bioassay tests that, at least in part, determine the water quality guidelines that have been established to safeguard aquatic habitats.

In the future, a more integrated approach that attempts to link laboratory and field data should be adopted for toxicity testing. For example, toxicity could be related to an internal metal content rather than an external metal concentration. Researchers would then be able to compare field uptake data with laboratory toxicity data in specific organisms. In addition, since internal toxicity appears to be independent of test conditions, this approach would circumvent the difficulties associated with determining metal speciation. Further research is required, however, to determine if the toxicity of internal metal loads varies with length of exposure or other factors.

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APPENDIX A. DATA

This appendix contains all the data that are presented and analyzed in the body of the thesis. Tables are numbered from A.1 to A.6 and correspond to the six experimental chapters of the thesis. Abvbreviations used in column headings in the appendix are elaborated below;

µg Cd................ Internal Cd content in µg Cd/g dry wt.

µg/frond............. The average weight of one frond in µg.

µg Zn................. Internal Zn content in µg Zn/g dry wt.

% Ca................ Internal Ca content in g/100 g dry wt.

% N.................. Internal N content. These data were lost due to analytical error.

% P.................. Internal P content in g/100 g dry wt.

Ca.................... Nominal external Ca concentration in µM.

Cd 14................ Measured external Cd concentration in µM in the culture flask on the last day of culture (day 14).

Cd..................... Nominal external Cd concentration in µM.

Counting day........ The day on which the fronds were counted.

Day.................. The middle day of the 14 day growth period for a series of 68 cultures which spanned a period of almost 600 days.

Days................. Days exposure to 0.64 µM Cd.

DT.................... Doubling Time. The time in days it takes for L. trisulca to double the number of fronds. It equals 301/MR.

DW................ mg oven dry weight.

EDTA................ Nominal external EDTA concentration in µM.

Exp.................. The first chapter is organized into a series of twelve sequential experiments. These are listed in order and are explained in the text of chapter 2.

Fe..................... Nominal external Fe concentration in µM.

mg Zn.. .... Internal Zn content in mg Zn/g dry wt.

MR.................... Multiplication Rate.

MR C................ The multiplication rate in control cultures.

MR Cd.............. The multiplication rate in cultures containing Cd.

N...................... Nominal external N concentration in µM.

P...................... Nominal external P concentration in µM.

Pre Cd............... The concentration of Cd in µM in the 6 week preconditioned cultures.

RED................. MR-C/MR-Cd.

Rep.................. Treatment rows with the same number were carried out at the same time. Treatment rows with different numbers were carried out at a different time. The order of experiments was sequential from 1 to 3.

Zn 14................ Measured external Zn concentration in µM in the culture flask on the last day of culture (day 14).

Zn.................... Nominal external Zn concentration in µM.

Table A.1. Data from Chapter 2. Axenic culture of Lemna trisulca.

Exp	MR	DT	DW	μg/frond	Exp	MR	DT	DW	μg/frond
1	30	10.0	5.4	390	7	0	-----	-----	-----
1	36	8.2	5.8	280	7	0	-----	-----	-----
1	36	8.4	4.4	220	7	0	-----	-----	-----
2	57	5.2	47	580	8	84	3.6	192	290
2	57	5.3	42	530	8	92	3.3	313	280
2	57	5.4	39	540	8	82	3.6	213	350
3	74	4.0	72	290	9	155	1.9	117	260
3	72	4.2	63	310	9	161	1.9	145	270
3	77	3.9	66	240	9	156	1.9	121	260
4	78	3.8	90	290	10	153	2.0	104	250
4	72	4.2	72	340	10	151	2.0	113	290
4	76	3.9	80	290	10	143	2.1	94	310
5	71	4.2	70	240	11	161	1.9	137	250
5	69	4.4	63	250	11	161	1.9	134	250
5	72	4.2	74	240	11	163	1.8	135	240
6	80	3.8	125	240	12	174	1.7	72	260
6	81	3.7	127	220	12	182	1.7	88	250
6	79	3.8	114	240	12	182	1.7	93	270

Table A.2.1. Data from Chapter 3. The effect of calcium on *Lemna trisulca*.

Ca	MR	DT	DW	% Ca	µg/frond
880	117	2.6	37	3.00	285
880	130	2.3	47	3.75	240
880	93	3.2	18	3.11	300
440	125	2.4	42	2.38	250
440	119	2.5	40	2.20	292
440	120	2.5	42	1.95	294
220	150	2.0	97	1.60	261
220	148	2.0	89	1.45	255
220	142	2.1	79	1.26	274
110	149	2.0	90	1.44	245
110	143	2.1	82	1.33	274
110	133	2.3	71	1.42	321
27.5	138	2.2	61	0.84	237
27.5	145	2.1	77	0.86	236
27.5	140	2.1	70	0.80	252
6.9	50	6.0	12	0.48	800
6.9	48	6.3	12	0.61	857
6.9	54	5.6	13	0.56	765
1.7	0	-----	0	-----	-----
1.7	0	-----	0	-----	-----
1.7	0	-----	0	-----	-----

Table A.2.1. continued

Ca	MR	DT	DW	% Ca	µg/frond
0	0	-----	0	-----	-----
0	0	-----	0	-----	-----
0	0	-----	0	-----	-----

Table A.2.2. Data from Chapter 3. The effect of phosphorus on Lemna trisulca.

P	MR	DT	DW	% P	µg/frond
240	145	2.1	72	1.86	227
240	138	2.2	65	1.85	250
240	148	2.0	81	1.72	228
120	143	2.1	72	1.61	239
120	146	2.1	75	1.55	228
120	143	2.1	72	1.58	237
60	156	1.9	108	0.85	238
60	149	2.0	87	1.26	238
60	153	2.0	87	1.22	212
30	161	1.9	120	0.84	225
30	151	2.0	87	0.92	227
30	152	2.0	89	0.94	218

Table A.2.2. continued.

P	MR	DT	DW	% P	µg/frond
15	143	2.1	69	0.65	228
15	157	1.9	118	0.58	253
15	148	2.0	76	0.61	215
7.5	140	2.2	82	0.58	300
7.5	149	2.0	96	0.38	262
7.5	150	2.0	106	0.42	279
3.8	126	2.4	56	0.34	320
3.8	123	2.5	52	0.36	331
3.8	125	2.4	50	0.36	292
1.9	119	2.5	38	0.29	277
1.9	124	2.4	53	0.25	319
1.9	119	2.5	46	0.26	331
0.9	101	3.0	25	0.16	316
0.9	100	3.0	25	0.16	333
0.9	111	2.7	34	0.14	318
0.4	97	3.1	22	0.15	319
0.4	91	3.3	18	0.16	316
0.4	92	3.3	21	0.14	356
0	89	3.4	21	0.09	396
0	83	3.6	18	0.10	419
0	78	3.9	13	0.12	351

Table A.2.3. Data from Chapter 3. The effect of nitrogen on Lemna trisulca.

N	MR	DT	DW	% N	µg/frond
500	132	2.3	63		294
500	126	2.4	47		272
500	133	2.3	59		268
125	129	2.3	58		302
125	129	2.3	51		270
125	130	2.3	56		279
31	132	2.3	60		284
31	122	2.5	40		261
31	129	2.3	47		245
8	103	2.9	28		341
8	99	3.1	19		264
8	101	3.0	25		321
2	80	3.7	14		350
2	80	3.8	13		333
2	84	3.6	14		311
0	70	4.3	10		345
0	54	5.6	7		412
0	59	5.1	10		500

Table A 3. Data from Chapter 4. Cadmium toxicity and its interaction with Ca in *Lemna trisulca*.

Treatment									
Cd	Ca	rep	MR	DT	DW	% Ca	µg Cd	Cd 14	µg/frond
0	27.5	1	155	1.9	99	0.70	4	0	223
0	27.5	2	148	2.0	84	0.77	0	0	234
0	27.5	3	152	2.0	98	0.64	2	0	240
0	55	1	156	1.9	109	1.06	0	0	241
0	55	2	151	2.0	92	1.04	0	0	235
0	55	3	150	2.0	93	0.85	13	0	249
0	110	1	151	2.0	100	1.47	0	0	255
0	110	2	152	2.0	104	1.34	1	0	258
0	110	3	147	2.1	88	1.01	4	0	259
0	220	1	136	2.2	75	1.48	0	0	316
0	220	2	147	2.1	92	1.66	0	0	271
0	220	3	155	1.9	102	1.34	3	0	229
0	440	1	145	2.1	89	2.36	0	0	281
0	440	2	146	2.1	86	2.32	1	0	258
0	440	3	142	2.1	90	2.09	4	0	304
0.04	27.5	1	152	2.0	97	0.75	26	0.05	243
0.04	27.5	2	146	2.1	79	0.72	34	0.05	236
0.04	27.5	3	136	2.2	56	0.96	33	0.04	236
0.04	55	1	146	2.1	77	1.00	46	0.04	233
0.04	55	2	149	2.0	96	0.96	38	0.04	264
0.04	55	3	145	2.1	76	0.80	45	0.06	233

Table A.3. continued

Treatment									
Cd	Ca	rep	MR	DT	DW	% Ca	µg Cd	Cd 14	µg/frond
0.04	110	1	142	2.1	80	1.25	48	0.05	275
0.04	110	2	154	2.0	114	1.43	44	0.05	268
0.04	110	3	148	2.0	88	1.15	53	0.05	249
0.04	220	1	144	2.1	99	1.92	50	0.05	320
0.04	220	2	147	2.1	95	2.00	50	0.05	275
0.04	220	3	136	2.2	73	1.79	47	0.05	300
0.04	440	1	148	2.0	99	2.00	57	0.05	277
0.04	440	2	134	2.3	59	2.10	86	0.05	265
0.04	440	3	135	2.2	66	2.05	97	0.06	282
0.08	27.5	1	142	2.1	69	0.74	76	0.10	239
0.08	27.5	2	145	2.1	85	0.86	67	0.10	261
0.08	27.5	3	151	2.0	100	0.63	68	0.13	260
0.08	55	1	153	2.0	108	1.07	56	0.09	262
0.08	55	2	145	2.1	84	1.02	75	0.10	259
0.08	55	3	151	2.0	101	0.88	88	0.13	262
0.08	110	1	151	2.0	106	1.54	80	0.10	270
0.08	110	2	152	2.0	109	1.31	78	0.09	268
0.08	110	3	142	2.1	84	1.17	94	0.11	292
0.08	220	1	141	2.1	89	1.31	88	0.12	312
0.08	220	2	135	2.2	78	1.83	115	0.10	338
0.08	220	3	139	2.2	76	1.50	113	0.11	291

Table A.3. continued

Treatment									
Cd	Ca	rep	MR	DT	DW	% Ca	μg Cd	Cd 14	μg/frond
0.08	440	1	129	2.3	57	2.65	130	0.08	298
0.08	440	2	133	2.3	71	2.30	111	0.10	324
0.08	440	3	133	2.3	71	1.86	117	0.11	323
0.16	27.5	1	129	2.3	53	0.72	177	0.18	273
0.16	27.5	2	141	2.1	70	0.76	143	0.19	245
0.16	27.5	3	149	2.0	89	0.70	122	0.26	242
0.16	55	1	147	2.1	92	1.11	136	0.19	267
0.16	55	2	139	2.2	73	1.08	151	0.18	278
0.16	55	3	135	2.2	63	0.95	192	0.22	269
0.16	110	1	124	2.4	53	1.23	193	0.19	327
0.16	110	2	141	2.1	81	1.43	191	0.18	289
0.16	110	3	144	2.1	80	1.18	221	0.21	253
0.16	220	1	139	2.2	87	2.01	175	0.18	327
0.16	220	2	134	2.3	74	1.58	211	0.19	329
0.16	220	3	149	2.0	103	1.38	220	0.24	279
0.16	440	1	137	2.2	81	2.64	230	0.20	321
0.16	440	2	133	2.3	72	2.80	250	0.20	327
0.16	440	3	133	2.3	81	2.06	258	0.22	367
0.32	27.5	1	93	3.2	19	0.74	756	0.31	311
0.32	27.5	2	120	2.5	45	0.76	676	0.32	317
0.32	27.5	3	119	2.5	40	0.68	495	0.52	286

Table A.3. continued

Treatment									
Cd	Ca	rep	MR	DT	DW	% Ca	µg Cd	Cd 14	µg/frond
0.32	55	1	100	3.0	24	1.00	616	0.35	316
0.32	55	2	123	2.5	48	0.92	392	0.43	304
0.32	55	3	111	2.7	34	1.15	715	0.24	315
0.32	110	1	119	2.5	40	1.12	484	0.31	286
0.32	110	2	124	2.4	52	1.29	406	0.33	315
0.32	110	3	124	2.4	53	1.19	323	0.39	323
0.32	220	1	112	2.7	34	1.62	642	0.34	312
0.32	220	2	124	2.4	49	1.61	475	0.33	304
0.32	220	3	115	2.6	36	1.50	583	0.38	293
0.32	440	1	117	2.6	35	2.08	620	0.34	269
0.32	440	2	111	2.7	39	1.82	593	0.35	368
0.32	440	3	129	2.3	57	1.74	567	0.45	295
0.64	27.5	1	66	4.6	7	1.0	2000	0.63	280
0.64	27.5	2	68	4.4	8	0.9	2400	0.49	296
0.64	27.5	3	75	4.0	10	0.8	1600	0.72	294
0.64	55	1	66	4.6	7	1.0	1100	0.56	280
0.64	55	2	67	4.5	5	1.8	3400	0.61	192
0.64	55	3	79	3.8	13	1.1	2000	0.83	342
0.64	110	1	72	4.2	8	1.2	1000	0.51	258
0.64	110	2	77	3.9	9	1.4	2100	0.52	250
0.64	110	3	97	3.1	19	1.5	1400	0.74	279

Table A.3. continued

Treatment									
Cd	Ca	rep	MR	DT	DW	% Ca	µg Cd	Cd 14	µg/frond
0.64	220	1	83	3.6	14	1.6	1600	0.49	318
0.64	220	2	72	4.2	7	1.9	2200	0.60	226
0.64	220	3	91	3.3	20	1.6	1500	0.64	351
0.64	440	1	82	3.7	12	2.0	1300	0.81	286
0.64	440	2	77	3.9	12	1.7	1200	0.65	333
0.64	440	3	69	4.4	10	1.9	1900	0.89	357
1.28	27.5	1	54	5.6	5	0.4	1200	1.17	294
1.28	27.5	2	57	5.3	3	1.0	3200	1.15	158
1.28	27.5	3	60	5.0	6	1.0	2000	1.25	286
1.28	55	1	60	5.0	4	1.0	1000	1.31	190
1.28	55	2	59	5.1	3	1.0	1400	1.09	150
1.28	55	3	57	5.3	6	1.1	2100	1.14	316
1.28	110	1	62	4.9	4	1.5	1500	1.29	182
1.28	110	2	60	5.0	5	1.4	2400	1.33	238
1.28	110	3	64	4.7	6	1.7	2600	1.31	250
1.28	220	1	54	5.6	3	1.7	1800	1.27	176
1.28	220	2	57	5.3	5	1.4	2200	1.35	263
1.28	220	3	59	5.1	3	2.3	4100	1.19	150
1.28	440	1	67	4.5	4	2.5	2500	1.65	154
1.28	440	2	68	4.4	6	1.7	2200	1.48	222
1.28	440	3	64	4.7	4	3.8	4300	1.72	167

Table A.4. Data from Chapter 5. Zn toxicity and its interaction with Cd in Lemna trisulca.

Treatment									
Cd	Zn	rep	MR	DW	μg Cd	μg Zn	Cd 14	Zn 14	μg/frond
0	0.08	1	183	127	0	59.8	0	-----	270
0	0.08	2	188	138	0.07	58.5	0	-----	257
0	0.08	3	175	95	0	65.3	0	-----	249
0	3.06	1	171	92	0	1250	0	2.72	269
0	3.06	2	171	82	0	1380	0	2.91	240
0	3.06	3	164	65	0	1660	0	3.09	235
0	6.12	1	142	37	0.57	6080	0	5.40	247
0	6.12	2	144	32	0	6310	0	5.58	201
0	6.12	3	152	39	0	6970	0	5.76	195
0	12.2	1	65	3.6	0	9800	0	12.4	200
0	12.2	2	63	4.7	0	10000	0	11.8	276
0	12.2	3	56	2.6	0	9120	0	13.1	186
0.16	0.08	1	156	71	129	74.2	0.15	-----	320
0.16	0.08	2	167	98	92.0	87	0.18	-----	320
0.16	0.08	3	159	72	103	106	0.15	-----	295
0.16	3.06	1	165	72	81.8	1400	0.15	2.72	250
0.16	3.06	2	170	88	52.6	1360	0.18	2.72	267
0.16	3.06	3	167	72	63.9	1690	0.15	3.09	240
0.16	6.12	1	145	39	136	6360	0.16	5.40	238
0.16	6.12	2	146	28	136	7610	0.19	5.76	165
0.16	6.12	3	127	21	162	7950	0.17	6.12	208

Table A.4. continued.

Treatment									
Cd	Zn	rep	MR	DW	µg Cd	µg Zn	Cd 14	Zn 14	µg/frond
0.16	12.2	1	61	3.1	321	9130	0.15	11.8	194
0.16	12.2	2	63	3.6	350	9800	0.20	12.4	212
0.16	12.2	3	50	1.8	255	9890	0.18	13.4	150
0.32	0.08	1	124	38	376	126	0.29	-----	409
0.32	0.08	2	126	40	428	190	0.34	-----	412
0.32	0.08	3	130	42	252	114	0.28	-----	389
0.32	3.06	1	161	69	156	1590	0.29	2.72	267
0.32	3.06	2	164	66	139	1130	0.34	2.91	236
0.32	3.06	3	158	61	161	1920	0.31	3.09	261
0.32	6.12	1	129	23	420	7740	0.29	5.76	215
0.32	6.12	2	126	14	498	14400	0.34	5.93	143
0.32	6.12	3	124	16	364	9690	0.30	6.30	172
0.32	12.2	1	74	4.5	658	8110	0.29	12.0	196
0.32	12.2	2	65	3.2	694	8500	0.35	12.4	178
0.32	12.2	3	53	2.1	652	9050	0.32	13.3	162

Table A.5.1. Data from Chapter 6. The effect of EDTA on Cd uptake and toxicity in *Lemna trisulca*.

Treatment								
Cd	EDTA	rep	MR	RED	DW	µg Cd	Cd 14	µg/frond
0	0	1	128	0.65	13	1.92	0	206
0	0	2	122	0.63	11	2.27	0	216
0	0	3	151	0.89	32	0.62	0	250
0	9	1	198	1	151	0.63	0	256
0	9	2	193	1	141	2.94	0.18	281
0	9	3	170	1	66	0.06	0	278
0	36	1	199	1.01	160	0.04	0	262
0	36	2	198	1.03	153	0	0.25	260
0	36	3	160	0.94	49	0	0	280
0	144	1	167	0.84	75	0.99	0.05	348
0	144	2	165	0.86	71	0	0.05	350
0	144	3	134	0.79	27	0	0	360
0.32	0	1	60	0.30	1.2	3000	35.3	135
0.32	0	2	64	0.32	1.2	3600	34.6	150
0.32	0	3	50	0.29	0.6	2300	41.0	120
0.32	9	1	133	0.67	28	443	36.0	384
0.32	9	2	138	0.72	29	397	33.2	333
0.32	9	3	97	0.57	8.4	1030	45.1	365
0.32	36	1	186	0.94	109	6.56	38.1	270
0.32	36	2	196	1.02	145	15.3	32.3	264
0.32	36	3	156	0.92	46	10.9	42.1	297

Table A.5.1. continued.

Treatment								
Cd	EDTA	rep	MR	RED	DW	µg Cd	Cd 14	µg/frond
0.32	144	1	170	0.86	80	0.48	30.2	336
0.32	144	2	176	0.91	93	1.9	40.0	314
0.32	144	3	133	0.78	25	1.56	31.4	347
1.28	0	1	34	0.17	0.8	1300	132	267
1.28	0	2	35	0.18	0.7	2500	138	233
1.28	0	3	34	0.20	0.3	3800	144	100
1.28	9	1	74	0.37	2.6	2500	148	236
1.28	9	2	82	0.42	4.4	1200	149	314
1.28	9	3	65	0.38	1.8	3300	159	225
1.28	36	1	185	0.93	116	55.6	133	302
1.28	36	2	190	0.98	125	50.6	117	276
1.28	36	3	162	0.95	53	46.8	136	282
1.28	144	1	164	0.83	65	3.31	132	333
1.28	144	2	185	0.96	114	9.21	120	292
1.28	144	3	133	0.78	26	4.92	117	356

Table A.5.2. Data from Chapter 6. The effect of Fe on Cd uptake and toxicity in *Lemna trisulca*.

Treatment						
Cd	Fe	MR	DW	μg Cd	Cd 14	μg/frond
0	9	160	59	0	0	235
0	9	163	64	0	0	235
0	9	158	56	0	0	240
0	2.25	156	54	0	0	241
0	2.25	160	65	0	0	259
0	2.25	152	54	0	0	273
1.28	9	40	2.5	2900	1.34	278
1.28	9	47	2.4	3800	1.28	218
1.28	9	31	1.8	3500	1.29	257
1.28	2.25	154	65	113	1.30	304
1.28	2.25	148	57	96	1.33	315
1.28	2.25	158	74	108	1.31	315

Table A.5.3. Data from Chapter 6. The effect of EDTA and Fe on Zn uptake and toxicity in Lemna trisulca.

Treatment								
EDTA	Fe	Zn	rep	MR	DW	mg Zn	Zn 14	μg/frond
9	9	0.08	1	160	69	0.15	-----	279
9	9	0.08	2	146	53	0.09	-----	316
9	9	0.08	3	152	40	0.14	-----	270
9	9	6.12	1	116	15	12	5.35	200
9	9	6.12	2	127	25	7.3	5.20	250
9	9	6.12	3	88	6.4	6.7	5.81	221
9	9	24.5	1	0	0.6	-----	18.3	200
9	9	24.5	2	10	0.7	-----	19.9	175
9	9	24.5	3	0	0.5	19	22.9	167
9	2.25	0.08	1	152	54	0.08	-----	271
9	2.25	0.08	2	151	58	0.05	-----	299
9	2.25	0.08	3	141	53	0.07	-----	262
9	2.25	6.12	1	136	29	-----	5.81	223
9	2.25	6.12	2	129	28	6.5	5.96	264
9	2.25	6.12	3	82	7.2	6.8	6.27	212
9	2.25	24.5	1	0	0.5	-----	24.5	167
9	2.25	24.5	2	0	0.5	-----	24.5	167
9	2.25	24.5	3	0	0.7	30	27.5	233
36	9	0.08	1	152	62	0.15	-----	308
36	9	0.08	2	153	59	0.03	-----	285
36	9	0.08	3	145	50	0.04	-----	303

Table A.5.3.continued.

Treatment								
EDTA	Fe	Zn	rep	MR	DW	mg Zn	Zn 14	µg/frond
36	9	6.12	1	157	65	0.37	6.12	283
36	9	6.12	2	150	47	0.39	6.27	246
36	9	6.12	3	149	52	0.21	6.42	284
36	9	24.5	1	114	20	9.7	26.0	282
36	9	24.5	2	120	19	11	27.5	229
36	9	24.5	3	135	41	3.8	27.5	331
144	9	0.08	1	117	22	0.26	-----	290
144	9	0.08	2	105	17	0.09	-----	309
144	9	0.08	3	113	19	0.09	-----	279
144	9	6.12	1	114	18	0.48	6.12	254
144	9	6.12	2	131	23	0.19	6.12	207
144	9	6.12	3	94	7.8	0.55	6.42	195
144	9	24.5	1	142	40	0.34	27.5	265
144	9	24.5	2	126	31	0.28	27.5	316
144	9	24.5	3	93	10.4	0.41	29.1	267

Table A.6.1. Data from Chapter 7. The lag between exposure and toxicity in *Lemna trisulca*.

Days	DW	µg Cd	µg/frond	Days	DW	µg Cd	µg/frond
0	156	0.5	286	8	54	1590	358
0	186	3.42	282	8	55	2340	342
0	193	0.7	264	8	83	1720	340
2	168	1170	315	10	24	2180	320
2	153	900	319	10	24	2440	338
2	156	1000	352	10	15	3370	288
4	127	1090	305	12	7.7	3920	285
4	108	828	327	12	8	3650	276
4	141	647	290	12	6.2	3940	258
6	89	1170	311	14	9.9	2100	430
6	95	1710	285	14	4.4	3000	338
6	87	1900	325	14	6.1	2980	339

Table A.6.2. Data from Chapter 7. The lag between exposure and toxicity in *Lemna trisulca*. Frond number.

	Counting day							
Days	0	2	4	6	8	10	12	14
0	1	3	7	17	41			546
0	1	3	8	20	49			660
0	1	3	8	20	51			731
2	1	3	8	18	40			534
2	1	3	8	20	45			480
2	1	3	8	18	37			443
4	1	3	8	20	51			416
4	1	3	7	19	46			330
4	1	3	8	22	51			487
6	1	3	8	19	49			286
6	1	3	8	20	49			333
6	1	3	7	19	44			268
8	1	3	8	19	43			151
8	1	3	8	19	42			161
8	1	3	8	20	40			244
10	1	3	8	15	30	39		75
10	1	3	7	17	22	41		71
10	1	3	8	14	22	40		52
12	1	3	7	8	16	18	22	27
12	1	3	7	9	17	19	22	29
12	1	3	7	8	12	17	19	24
14	1	3	3	7	8	13	15	23
14	1	3	3	5	6	8	12	13
14	1	3	4	6	6	12	13	18

Table A.6.3. Data from Chapter 7. Induction of Cd tolerance in *Lemna trisulca*.

Cd	Pre Cd	MR	DW	µg Cd	Cd 14	µg/frond
0	0	154	57	0	0	266
0	0	143	40	0	0	253
0	0	156	52	0	0	234
0	0.08	133	37	0	0	311
0	0.08	149	44	0	0	242
0	0.08	152	50	0	0	253
0	0.32	153	50	0	0	242
0	0.32	129	37	0.60	0	346
0	0.32	155	59	1.25	0	273
0.16	0	140	44	219	0.14	308
0.16	0	120	30	160	0.20	361
0.16	0	135	35	171	0.17	280
0.16	0.08	111	20	284	0.16	313
0.16	0.08	119	24	198	0.21	296
0.16	0.08	139	41	152	0.19	293
0.16	0.32	141	43	157	0.14	295
0.16	0.32	114	25	219	0.19	357
0.16	0.32	136	42	144	0.20	323
0.32	0	69	6.3	537	0.27	315
0.32	0	110	22	468	0.32	349
0.32	0	83	12	795	0.33	400

Table A.6.3. continued

Cd	Pre Cd	MR	DW	μg Cd	Cd 14	μg/frond
0.32	0.08	83	12	671	0.28	400
0.32	0.08	113	20	497	0.37	294
0.32	0.08	113	24	471	0.35	348
0.32	0.32	118	32	366	0.26	405
0.32	0.32	87	15	673	0.38	455
0.32	0.32	106	21	628	0.34	375
0.64	0	40	2.9	1780	0.56	322
0.64	0	56	3.7	3030	0.77	264
0.64	0	56	4.3	1670	0.78	307
0.64	0.08	50	4.6	1780	0.58	383
0.64	0.08	69	6.4	1430	0.85	320
0.64	0.08	67	6.6	1670	0.79	347
0.64	0.32	70	7.7	1610	0.48	367
0.64	0.32	56	5.2	1960	0.81	371
0.64	0.32	72	7.3	1320	0.72	332

Table A.6.4. Data from Chapter 7. The effect of intrinsic multiplication rate of Lemna trisulca on Cd toxicity. MR over time.

day	MR	day	MR	day	MR
8	144	262	152	431	130
8	142	262	132	431	129
8	134	262	139	431	140
29	134	262	139	500	146
29	148	269	150	500	147
29	139	277	164	505	137
99	140	283	162	505	143
99	134	295	174	506	145
99	125	295	180	506	152
129	142	295	183	506	146
142	132	358	176	506	154
142	136	360	171	506	156
142	128	379	169	527	141
151	143	386	174	527	139
169	138	395	163	534	150
191	140	402	148	534	153
198	140	409	146	534	148
207	145	409	150	554	150
228	139	409	144	561	138
242	151	417	141	570	143
262	147	417	140	577	145
262	150	417	140	589	136

Table A.6.5. Data from Chapter 7. The effect of intrinsic multiplication rate on Cd toxicity in *Lemna trisulca*.

MR C	MR Cd	RED	MR C	MR Cd	RED
155	93	0.40	193	138	0.28
148	120	0.19	170	97	0.43
152	119	0.22	191	141	0.26
156	100	0.36	195	142	0.27
151	123	0.19	184	145	0.21
150	111	0.26	155	119	0.23
151	119	0.21	145	116	0.20
152	124	0.18	145	109	0.25
147	124	0.16	145	108	0.26
136	112	0.18	145	122	0.16
147	124	0.16	145	130	0.10
155	115	0.26	145	122	0.16
145	117	0.19	145	128	0.12
146	111	0.24	148	102	0.31
142	129	0.09	166	93	0.44
198	133	0.33	157	128	0.18
			168	106	0.37

APPENDIX B. GLOSSARY OF TERMS

ACS................... American Chemical Society.
ANOVA............. Analysis of variance.
CDTA................. Trans-1, 2-cyclohexylene dinitrilotetraacetic acid.
DT..................... Doubling time (301/MR).
EC50.................. A toxicant concentration which reduces a parameter to 50% of a control value.
EDDHA.............. Ethylenediamine-N,N'-bis-(o-hydroxyphenylacetic acid).
EDTA................. Ethylenediaminetetraacetic acid.
FA..................... Fulvic acid.
HA..................... Humic acid.
HEDTA.............. N-(2-hydroxyethyl) ethylenedinitrilo-N, N', N' -triacetic acid.
HEPES................ N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
LOEC................. Lowest observable effect concentration.
μM.................... Micromoles/liter.
$\mu mol/m^2/s$ PAR..... Micromoles/meter^2/second photosynthetically active radiation.
MR.................... Multiplication rate.
nM..................... Nanomoles/liter.
Negative control..... A control which contains no toxicant.
NOEC................. No observable effect concentration.
NOSTASOT.......... No statistical significance of trend.
NTA................... Nitrilotriacetic acid.
PC..................... Phytochelatins.
pH..................... Negative Log [H^+ activity].
PPB................... Parts per billion (μg/liter).
PPM................... Parts per million (mg/liter).
Positive control...... A control which contains a standard amount of reference toxicant.
sd...................... Standard deviation.
SDS................... Sodium dodecyl sulphate.