POPULATION DIFFERENCES IN THE TOXIC EFFECTS OF HEAVY METALS TO *LITTORINA SAXATILIS* OLIVI (PROSOBRANCHIA: MOLLUSCA)

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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To Ma and Pa

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Population Differences in the Toxic Effects of Heavy Metals to *Littorina saxatilis* Olivi (Prosobranchia: Mollusca)

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ABSTRACT

Mine derived heavy metal contamination has been reported from estuaries in the Isle of Man that are associated with historical mining regions. Current levels of zinc (Zn), lead (Pb), copper (Cu) and cadmium (Cd) in sediment indicate Zn and Pb are highly elevated in the Peel and Laxey estuaries in comparison with the Castletown and Ramsey estuaries. Metal levels in *Mytilus edulis* and *Fucus serratus* also indicate a similar pattern of contamination. The relative levels of metals in the two species show that 'total' Zn levels were higher in the Peel estuary than Laxey but that as result of physico-chemical differences between the two sites, there was more soluble Zn at Laxey. *Littorina saxatilis* and *Enteromorpha intestinalis* (from shore level of *L. saxatilis*) showed higher metal burdens in the Laxey Estuary except for Cu in *L. saxatilis*. Zn values in the winkles from Laxey was significantly higher than individuals from other sites including Peel. Relatively low metal values were obtained in *L. saxatilis* from Peel in comparison with other indicators and this is thought to reflect site specific ecological differences related to height of sample collection.

Acute toxicity experiments in the laboratory indicated that the population of *L. saxatilis* in the Laxey estuary was more tolerant to Zn and Pb than those from Derbyhaven, Castletown, Peel and Ramsey. At 10 mg l^{-1} Zn and 5 mg l^{-1} Pb, individuals from Laxey showed significantly lower susceptibility to these metals as reflected in LT_{50} values. Time mortality curves for exposures to 5mg l^{-1} Zn at which mortalities for individuals from Laxey were much lower than 50 % also indicated a similar results but at 20 mg l^{-1} Zn minimal differences in mortality were found. There appeared to be no co-tolerance to Cd and Cu.

Experimental exposures to acute ecological stress (0 psu and 60 psu salinities, and aerial exposure at 24 °C) showed that there might be some physiological 'trade-offs' to metal tolerance in *L. saxatilis*. This was demonstrated in response to desiccation but not to either hyper- or hyposaline stress.

Inter-site dfferences in metal accumulation from solution showed that tolerance to Zn was achieved by reduced net uptake of the metal by tolerant individuals. Significantly lower regression slopes were obtained for the accumulation of Zn at 2.5mg l^{-1} and 5mg l^{-1} over a six-day exposure period. However, Pb tolerance appeared to be the result of the ability of tolerant individuals to sequester the metal and 'detoxify' it in the tissue as the winkles from Laxey had a significantly higher rate of accumulation. Zn and Pb accumulation from mixtures of the metals in solution indicated that tolerances to the two metals were more likely to be independent than linked.

There appear to be some reproductive effects of heavy metals on *L. saxatilis* in the Laxey Estuary. This is reflected in lower sizes of juveniles at birth. A higher absolute number of embryos in the brood pouches of individuals from Laxey is presumed to be a response to mitigate the direct or indirect effects of heavy metals on juvenile mortality. No significant size-specific differences in embryo numbers were observed. There was a high intra-site variability in the number of embryos and the observed differences in absolute counts could not be unequivocally ascribed to pollution effects. No heavy metal-related differences in the proportions of embryo abnormality was apparent from my results. The highest proportion of abnormal embryos was obtained from Derbyhaven where the levels of the four metals measured in this study from seaweed and the winkles were not elevated. The cause is unknown but it may be related to disease, genetic factors or unknown toxins.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

Over the last 30 years, there has been much concern over the potential danger of conservative pollutants such as pesticides and heavy metals in the aquatic environment and the possible risks to human health. Public awareness of the consequences of heavy metal pollution in the aquatic environment was attracted by the episodic Minamata mercury poisoning of the 1950's and the "itai itai" disease of the early 1960's (attributed to cadmium) in Japan (Gerlach, 1981).

The term "heavy metal" (used synonymously with "trace metal") refers to most metals and metalloids with the exception of the alkali, alkaline earths, lanthanides and actinides (Förstner & Wittman, 1979). Unlike most other contaminants, they are natural constituents of sea water derived from geochemical and volcanic processes. Rivers are a major contributor of heavy metals to the sea, the nature of input depending on the occurrence of metal and ore bearing deposits in the drainage area (Watling & Emmerson, 1981; Clark, 1992). The sources of anthropogenic input of heavy metals into the aquatic environment include industrial processes (Buckley *et al.*, 1995; Chen & Wu, 1995; Shear *et al.*, 1996), sewage sludge (Blomqvist *et al.*, 1992; Birch & Davey, 1995), power stations (Philips & Unni, 1991) and mining (Davies, 1987; Porvari, 1995). The mining of heavy metals by man is increasing the mobilization of most elements over that achieved by natural geological weathering. Metals such as zinc and copper are being mined and mobilized at rates over ten times those expected from simple geological processes of weathering (Phillips, 1980). Even when mining has stopped, drainage water from spoil heaps is a continuous source of contamination (Bryan, 1976a; Bryan & Gibbs, 1983; Southgate *et al.* 1983; Hunt & Howard, 1994).

A lot of industries discharge one trace metal or the other into water or soil (Nriagu & Pacyna, 1988). Examples of industrial processes which utilize selected heavy metals are given in Table 1.1. Rivers and freshwater run-off constitute a major route of entry of metals into estuaries and the sea (Bryan, 1976a). The atmosphere is also an important route of entry of heavy metals into estuaries and the sea (Vale & Harrison, 1994). Atmospheric processes involved in metal transport are still poorly understood and estimates of inputs to the sea may vary widely due to difficulties in sampling and analysis (Langston & Spence, 1994). However, Preston (1992) states that anthropogenically derived trace metals in the atmosphere are predominantly within aerosols (with mercury being a significant exception) and are distinguished by having a high concentration relative to the average crustal abundance. Nriagu & Pacyna (1988) reported that the average anthropogenic emission of arsenic, cadmium, copper, nickel, and zinc exceed inputs of these elements from natural sources by two fold or more. In the case of lead, the atmosphere has been recognized as a major route of entry into natural waters (Flegal & Patterson, 1983 ; Veron *et al.*, 1987; Lambert *et al.*, 1991a) and the ratio of anthropogenic to natural emission can be as high as 17 (Nriagu & Pacyna, 1988). Atmospheric contaminants are deposited by gas exchange at the sea surface; the fall-out of particles (i.e. dry deposition), or as precipitations in rain- or snow- fall (i.e. wet deposition) (Förstner and Wittmann 1979; Clark, 1992; Langston & Spence, 1994). The balance between the relative significance of wet and dry depositional processes is a function of particle size, geographical location and associated weather conditions (Lambert *et al.*, 1991b; Preston, 1992) .

A distinction is usually made between essential and non-essential heavy metals on the basis of known biological functions. Essential elements include zinc (Zn), copper (Cu),

Table 1.1 Some industrial uses of selected heavy metals.

Metal	Industrial Use
Lead	Storage batteries, metal products, pigments, auto and boat fuel, ammunition.
Zinc	Galvanising iron and steel products and alloys.
Copper	Electrical, automotive, construction, plumbing, antifouling paints.
Cadmium	Electroplating, pigments in plastic industry, ceramics, paint coatings, photography.
Mercury	Insecticides, fungicides, herbicides, bactericides, pharmaceuticals, manufacture of chlorine, electronics.
Arsenic	Plant desiccants, poultry and food additives, pharmaceuticals and detergents.
Nickel	Numerous uses as an alloy in combination with Fe and C (nickel-steel, plantenite), Cr & Zn (German silver) and others such as Mn, Al and Mo.
Silver	Electroplating, food and beverge processing.
Chromium	Metal plating, industrial dyes, ink.
Selenium	Smelting of copper.
Tin	Antifouling paints.

Source: compiled from Higgins & Burns, 1975; Forstner & Wittman, 1979; Moore & Romamoorthy, 1984.

iron (Fe), molybdenum (Mo), manganese (Mn), chromium (Cr), cobalt (Co), vanadium (V), selenium (Se), nickel (Ni) and tin (Sn). These elements are vital components of enzymes and respiratory pigments (White & Rainbow, 1985). For example, haemoglobin contains Fe; Haemocyanin contains Cu; carbonic anhydrase, carboxypeptidase A and B and several dehydrogenases contain Zn; Pyruvate carboxylase contains Mn; vitamin B_{12} enzymes contain Co; xanthine oxidase contains Fe and Mo; and cytochrome oxidase contains Fe and Cu (Bryan, 1976a). Other metals such as mercury (Hg), cadmium (Cd) and silver (Ag) have no known biological function (Bryan, 1984). Whether essential or not, heavy metals exert an adverse effect on organisms when present in the environment in elevated concentrations. The balance between requirement and toxicity of metals is delicate and depends on a variety of abiotic and biotic factors (White & Rainbow, 1985).

Because mortality is the most obvious effect of a pollutant and is relatively easily determined in most organisms, it is the most commonly measured end-point in aquatic organisms. Acute toxicity tests are useful in establishing critical concentrations, usually LC_{50}s (the concentration that kills 50 % of test animals in a given time) to set emission levels (Widdows, 1993). Richardson & Martin (1994) state that the toxicity test approach to surveillance and monitoring of inputs to marine and estuarine environments has been the "cornerstone" of regulatory procedures. LC_{50} estimations have been done for several metals in many invertebrate species and fishes (e.g. Cd in *Agropecten irradians,* Pesch & Stewart, 1980; Cu, Ni and Zn in *Crassostrea virginica,* Calebrese *et al.*, 1973; Zn in *Mytilus edulis,* Ahsanullah, 1976; see Mance, 1987 for reviews).

Enhanced tolerance to lethal concentrations of heavy metals has been reported to occur in fish and invertebrates pretreated with sublethal concentrations of the metals (e.g. Sinley *et al.*, 1974; Bouquegneau, 1979; Pesch & Hoffman, 1982; Roesijadi *et al.*, 1982; Roesijadi & Fellingham, 1987). Tolerance has also been demonstrated in some aquatic species from areas of ambient metal contamination (e.g. *Ectocarpus siliculosus*, Russel & Morris, 1970; *Acartia clausi*, Moriatou-Apostolopoulou, 1978, Luoma *et al.*, 1983; *Carcinus maenas*, Bryan & Gibbs, 1983; *Asellus meridianus*, Brown, 1976, 1977, 1978; *Nereis diversicolor*, Bryan & Hummerstone, 1971, Hately *et al.*, 1989; *Mytilus edulis*, Hvilson, 1983; and *Littorina saxatilis*, Robinson 1985, Webb, 1990).

Tolerance to acute metal levels may result from genetic selection (genotypic) which might be expressed phenotypically; or through a mere adjustment of biochemical and physiological mechanisms (phenotypic) without a genetic basis. Although evidence for the basis of metal tolerance in most aquatic species is not conclusive, the existence of genetically selected strains has been clearly shown in some species. *Nereis diversicolor* from Restronguet Creek, Cornwall has been reported to belong to a tolerant strain (Bryan & Hummerstone, 1971; Hately *et al.*, 1989), a feature that has also been used in mapping the ecological impact of metals on the species by Grant *et al.* (1989). Similarly, Brown (1976) reported that tolerance to Pb by *Asellus meridianus* from the Gannel persisted in a laboratory reared F_2 generation.

1.2 AIMS OF STUDY

Mine-derived contamination by Zn and Pb has been reported for sediment and biota from estuaries in the Isle of Man that are associated with a history of mining: Laxey and Peel estuaries had high concentrations of metals in comparison with Castletown and Ramsey (Southgate *et al.*, 1983).The published values are for samples obtained in 1979, about seventeen years ago. Gibb *et al.* (1996) have recently reported elevated concentrations of metals in some biomonitors but no recent records of sediment metal loadings are available. In chapter 2 the sites under study are briefly outlined. The initial aim of this work was to assess the current level of contamination by heavy metals in these sites using sediment and selected biomonitors (chapter 3).

The development of metal tolerance in populations pre-exposed to metals under laboratory and field conditions has been established for several species. In making a choice of an organism for tolerance studies from an environment that has had a long-term exposure to metals, it is advantageous to select one that lacks a larval stage. *Littorina saxatilis* was chosen for this work because it is ovoviviparous. The second part of chapter 2 is a brief review of its biology and much debated taxonomy. As a result of the absence of a larval stage, there is a limited dispersal of young which favours the formation of discrete populations (Janson,1982; Janson & Ward, 1984; Johannesson *et al.*, 1993) with the potential of developing metal tolerance. Differences in metal tolerance in *L. saxatilis* from the various sites were tested (chapter 4).

Metal tolerance may be a mere expression of general stress tolerance in a population. On the other hand, it may be achieved at a "cost" which could pre-dispose metal tolerant populations to other stressors. Tolerance to general stressors (salinity and desiccation) was therefore tested (chapter 5).

The mechanism of metal tolerance was investigated by studying differential uptake of metals (chapter 6).

Reproductive effects may occur in polluted sites even if adult animals appear to thrive (Dixon, 1983, Dixon & Pollard, 1985). Differences in fecundity, embryo abnormalities and size at birth of young were assessed (chapter 7).

The general discussion (Chapter 8) gives an overview of the work and highlights the limitations.

CHAPTER TWO

STUDY SITES AND TEST ORGANISM

2.1 STUDY SITES

The Isle of Man has a long history of mining and the earliest record of mining dates back to the 13th century (Lamplugh, 1903). Although little is known about the earliest mine workings, archaeological evidence from excavations at Kiondroghad suggests that iron ore was being obtained in the Dark ages (Gerrad *et al.*, 1972). Large-scale exploitation started in the 18th century and peak production was attained in the mid 19th century. This was followed by a steep drop in production, such that the last mine closed in 1919, although some work was carried out from 1922 and 1929. Between 1953 and 1957, the Island Exploration Company formed with the support of the Manx government undertook a comprehensive survey aimed at reviving old mines and discovering new mineralised areas. A considerable amount of geochemical and geophysical prospecting was done, culminating in a short-lived drilling programme which produced no positive indication of economically viable orebodies (Mackay & Schnellmann, 1963).

The predominant ores were sulphides of zinc, lead and copper; small amounts of silver associated with the lead ore and traces of gold were also found. The distribution of mineral veins and mines (Fig. 2.1) suggests that zinc, lead, copper and iron were present in small amounts throughout the Manx Slate (a largely lower ordovician age formation covering about two thirds of the island). However, the two main groups of mines were at Laxey and Foxdale. The Foxdale mines were predominantly galena, mostly argentiferous, whilst at Laxey sphalerite was the chief ore. The Laxey mines produced zinc, copper and lead while at Foxdale lead was mainly produced (zinc being present in uneconomical quantities). In 1884-85, the Laxey mines produced more zinc

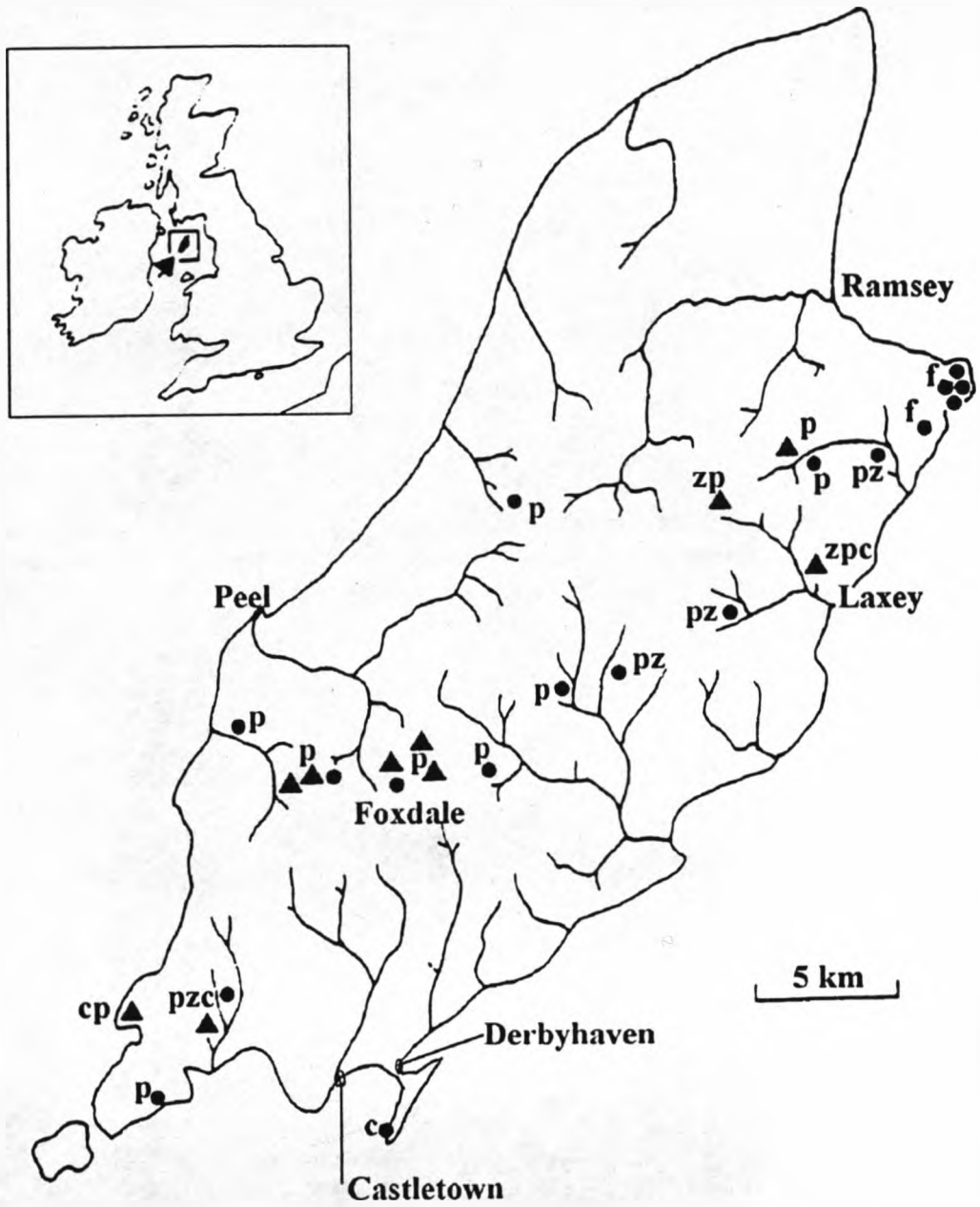

Fig 2.1 Locations of sampling sites and the distribution of mines around the Isle of Man. Filled triangles and circles represent major and minor producing mines respectively and the order of importance of the ores produced is indicated (c = copper, f = iron. p = lead, z = zinc).
Distributions of mines and ores produced, after Southgate *et al.*, 1983.

blende than the combined output of all the other mines in the British Isles, although in lead it was surpassed by Foxdale (Gerrad *et al.*, 1972). Overall, the Manx output amounted to a fifth of all the zinc ever produced in the British Isles, and perhaps as much as 5 % of the lead (Ford, 1993).

Although mining stopped a long time ago, there are still spoil heaps and adits leading from the mines. These are capable of contributing to elevated metal levels in rivers (Abdullah & Royle, 1972). Elevated amounts of zinc and lead have been reported in sediments of rivers draining areas of past mining activity in the Isle of Man (Southgate *et al.*, 1983). Similar observations have been made in river metal loadings in both dissolved and particulate phases (Atkinson, pers. comm.). The freshwater input to the estuaries at Laxey and Peel come from rivers that drain the main mining areas of Laxey and Foxdale respectively and metal levels in sediments and biota from these estuaries are much higher than those of Castletown and Ramsey (Southgate *et al.*, 1983, Gibb *et al.*, 1996).

The contamination status of estuaries described above was the basis of selection of sites for this study. The long-term exposure of populations in contaminated estuaries may have resulted in the selection of metal-tolerant populations. *Littorina saxatilis* were collected from five sites (Fig. 2.1): the esturarine harbours at Laxey (Grid ref: SC444836), Peel (SC242846), Castletown (SC266675) and Ramsey (SC455946), and at Derbyhaven (SC284671) (a coastal rocky shore with very high abundance of *L. saxatilis*).

2.2 TEST ORGANISM: *Littorina saxatilis*

The rough periwinkles, commonly referred to as *Littorina saxatilis* species complex are of variable size and colour, and occupy areas of the shore (sheltered to very exposed) from the lower littoral fringe to the upper eulittoral (Hawkins & Jones, 1992). They are widespread in distribution, ranging from the Mediterranean along all European shores to the Arctic seas; and in the Western Atlantic, components of the complex extend south to the Delaware Bay and in the Eastern Pacific to Puget Sound (Fretter & Graham, 1980). They feed on the surface layers of the weed on which they live, green filamentous algae, and on the biofilm on rocks primarily composed of unicells and detritus (Fretter & Graham, 1980, 1994; Norton *et al.*, 1990). It is difficult to distinguish between the sexes on the basis of external shell characteristics, but mature females and males can be identified by the examination of the reproductive structures (Reid, 1993).

The taxonomic history of the *Littorina saxatilis* complex has been the subject of much controversy and debate (see reviews by Fretter & Graham, 1980, 1994; Raffaelli, 1982; Reid, 1993). Four species are commonly recognized in the complex in current literature: *L. saxatilis* (Olivi, 1792), *L. neglecta* (Bean, 1884), *L. arcana* (Hannaford Ellis, 1978) and *L. nigrolineata* (Gray, 1839) (Warwick *et al.*, 1990; Reid, 1993). The first two are ovoviviparous (produce live young or 'crawlaways') and the latter two are oviparous (lay egg masses from which young hatch) (Warwick *et al.*, 1990). However, this classification does not command universal acceptance and some authors (e.g. Caugant & Bergerard, 1980; Hughes & Roberts, 1981) consider *L. arcana* as a

reproductive morph of *L. saxatilis* (=*L. rudis*); and Smith (1982) has suggested that a further form, *Littorina tenebrosa* should be given a specific rank.

Allozyme electrophoresis studies appear to suggest that there are only three taxa that deserve species status: *L. saxatilis*, *L. nigrolineata* and *L. arcana* with the status of *L. neglecta* being questionable (Johannesson & Johannesson, 1990 a, 1990b). They suggested that *L. neglecta* should be a junior synonym of *L. saxatilis*. The three true species are closely related; genetic distance (Nei's D) range from 0.035 to 0.083 which is in the lower range of genetic distances between congeneric animal species (Johannesson *et al.*, 1993).

Johannesson & Johannesson (1990a) found snails fitting earlier descriptions *of L. neglecta* from the barnacle zone in Port St. Mary ledges, Isle of Man but contend that the form is not clearly distinguishable from *L. saxatilis*. Neither Webb (1990) in a casual observation nor a review of 'saxatilid' winkles by Mill & Grahame (1990) found *L. arcana* in the Isle of Man. Also *L. arcana* is not usually found in estuaries (Mill & Grahame, 1990). *L. nigrolineata* tends to live at lower shore levels than other species of the *saxatilis* complex (Fretter & Graham, 1980) and is more easily distinguishable on external features. It is therefore suggested that the littorinids of the *saxatilis* complex sampled for this work were certainly virtually all *Littorina saxatilis*.

CHAPTER THREE

HEAVY METAL CONTAMINATION IN SEDIMENT AND BIOMONITORS FROM SITES AROUND THE ISLE OF MAN

3.1 INTRODUCTION

The contamination of an aquatic system by heavy metals can be measured by the analysis of water, sediment or certain organisms commonly termed "bio-indicators" (Bryan *et al.*, 1985) or "biomonitors" (Rainbow & Phillips, 1993).

Analysis of seawater is perhaps the most obvious way of assessing contamination (Bryan *et al.*, 1985) and there are numerous reports of the concentrations of heavy metals in water from open oceans, coastal areas or estuaries (e.g. Gill & Fitzgerald, 1985; Ferrara & Maserti, 1986, 1988). Metal concentrations are, however, normally very low and most samples require the pre-concentration of large volumes of water either by organic extraction or by resin chelation techniques (Phillips, 1977a; Tomlinson *et al.*, 1980; Langston & Spence, 1994). Apart from being expensive and laborious, the number of steps involved increases the possibility of either positive or negative contamination. Also, the determination of heavy metals in estuarine and marine waters is influenced by interference effects arising from the high salt content of the matrix (Langston & Spence, 1994). Initial interferences may be reduced by filtration of the water through 0.45µm, removing undissolved components (Hunt, 1986). The use of polarographic methods (Anodic Stripping Voltammetry-ASV e.g. Dabeka & Ihnat, 1987; or Cathodic Stripping Voltammetry-CSV e.g. VanDenBerg, 1986; VanDenBerg *et al.*, 1991) entails preliminary treatment of samples designed to eliminate interference from chlorides, turbidity, organic matter, oxidising and reducing substances and dissolved oxygen. ASV and CSV also divide metal species into two categories: electroactive (aquo ions and "labile" complexes) and electroinactive (organic complexes and colloidal species) (Förstner & Wittmann, 1979).

Tomlinson *et al.* (1980) contended that the greatest problem with water analysis as a means of comparing the degree of metal pollution in different locations is that the metals in this phase are more or less transitory. Substantial variations in concentration of metals occur temporally with differences in season (Boyden *et al.*, 1979; Morris, 1974; Johnson & Thornton, 1987; Kremling & Pohl, 1989; James *et al.*, 1993). On a shorter time scale, variations may occur with tidal cycle (Boyden *et al.*, 1979; Morris *et al.*, 1982; Valsaraj *et al.*; 1995; Jones, 1995) and extent of freshwater inflow (Windom *et al.*, 1983). More importantly, it is not always possible to predict toxicological effects in biota on the basis of total (or soluble) concentrations of metals in water. For instance, the most bioavailable dissolved forms of Cu, Cd, and Zn are the inorganic ions Cu^{++}, Cd^{++}, and Zn^{++} respectively, while for Hg the organic form, methylmercury, is by far more bioavailable than the inorganic species (Anderson *et al.*, 1978; Engel *et al.*, 1981; Bryan *et al.*, 1985; O'Brien *et al.*, 1990).

In comparison with water, the analysis of sediments is relatively easier because sediments are an accumulating sink, so heavy metal concentrations in sediments usually exceed those in the overlying water (Bryan *et al.*, 1985; Bryan & Langston, 1992). The relatively higher concentrations also benefit analytical precision, accuracy and monitoring efficiency (Hanson *et al.*, 1993). Heavy metals in sediments are a particularly useful indication of chronic contamination (Bryan *et al.*, 1985). Spatial continuity of contamination in surficial sediments can aid detection of contamination sources even after cessation of discharges (Hanson *et al.*, 1993). However, a number of factors render the interpretation of sediment metal data difficult. Large variations of heavy metal concentration occur in sediment because of differences in particle size distribution and nature (DeGroot *et al.*, 1976; Vaithiyanathan *et al.*, 1993), mineralogy (DeGroot *et al.*, 1976; Hanson *et al.*, 1993),

water content (Piron *et al.*, 1990), and the presence or absence of organic material (Phillips, 1977a; Fowler, 1990). Also, metal concentrations in sediment may bear little relation to the levels which are biologically available (Luoma, 1983; Bryan & Langston, 1992).

The use of biomonitors in monitoring aquatic pollution began some 30 years ago with studies of radionuclide abundance in marine ecosystems (Folsom *et al.*, 1963; Folsom & Young, 1965; Phillips & Segar, 1986). Analysis of organisms has obvious advantages over the use of water and sediment when determining the mechanisms and consequences of metal uptake, since tissue burdens are often a direct manifestation of biologically available metal in the environment (Zaroogian, 1980; Bryan *et al.*, 1985; Langston & Spence, 1994). In addition, they provide time-integrated measures of the levels of metal contamination (Rainbow & Phillips, 1993), and their ability to accumulate metals to high concentrations makes analysis relatively easy. The general acceptance of the advantages inherent in the use of biomonitors to monitor aquatic pollution has given rise to the establishment of national and international programmes employing such species in many parts of the world (Goldberg *et al.*, 1978,1983; Phillips & Segar, 1986; Phillips, 1989).

An ideal bio-indicator should satisfy certain criteria (see Phillips, 1980; Rainbow & Phillips, 1993; Langston & Spence, 1994). These include:

1. The organism should accumulate pollutants without being killed by the levels encountered in the environment.
2. The organism should be sedentary in order to be representative of the study area.

3. The organism should be sufficiently long-lived to allow the sampling of more than one year-class, if desired.

4. The organism should be of reasonable size, giving adequate tissue for analysis.

5. The organism should be easy to handle and identify, and hardy enough to survive in the laboratory to allow defecation before analysis (if desired) and laboratory studies of pollutant uptake.

6. The organism should tolerate brackish water to allow transplantation.

7. A simple correlation should exist between the pollutant content of the organism and the average pollutant concentration in the surrounding water.

A number of extrinsic (physico- chemical) and intrinsic (biological) factors, (reviewed by Phillips, 1980; Phillips & Rainbow, 1993), may introduce variability in the use of biomonitors. Physical and chemical parameters include variation due to microhabitat (Nielson, 1974; Roberts *et al.*, 1986); variation in salinity (George *et al.*, 1978) and temperature (Fischer, 1986); metal-metal/ligand interaction (Bryan, 1969; Jackim *et al.*, 1977; George & Coombs, 1977). The biological factors include differential metal-binding abilities between individuals and between tissues/organs of the same individual (Mason & Simkiss, 1983); size or age (Boyden, 1974; 1977; Watling & Watling, 1976); growth rate (Phillips, 1976b), reproductive stage (Fowler & Oregioni, 1976) and sex (Schulz-Baldes, 1974; Watling and Watling, 1976).

Since marine organisms take up heavy metals from a variety of sources (both from solution and particulates), accumulated metal concentrations of a single species will reflect sources appropriate to that particular organism. In effect, suspension feeders such as mussels, oysters and barnacles may ingest metals from detritus or plankton (Phillips, 1976a, 1976 b;

Zaroogian, 1980; Powell & White, 1990; Nolan & Dahlgaard, 1991). Sediment dwellers may take up metals released from sediment particles to interstitial water, at least where physico-chemical conditions in the sediment promote this (McGreer, 1979; Luoma & Bryan, 1982; Wright & Zamuda, 1987) or by ingestion of sediment particles (Bryan & Langston, 1992). Also, different metals have their individual characteristics which will influence their uptake by organisms (Nor, 1987; Tessier & Campbell, 1987,1990).

Some indicators are versatile and reflect contamination with a wide range of metals although not with equal efficiency, while others may be limited to a few (see Table 3.1; Bryan *et al.*, 1985). Clearly then, there is no universal bio-indicator of heavy metal pollution and it is appropriate to use a suite of species of different ecological type :(i) a macrophytic alga responding essentially to dissolved metal sources only (ii) a suspension feeder responding to sources of metal in both dissolved and suspended phase and/or (iii) a deposit feeder. A herbivore or carnivore may be added to obtain evidence of food chain biomagnification (Bryan *et al.*, 1985; Rainbow & Phillips, 1993).

In this chapter, I present the heavy metal levels in sediment and some biomonitors at selected sites around the Isle of Man with previous histories of mining and areas presumed to be suitable as "clean" control sites (see chapter 2.1). Biomonitors selected were *Mytilus edulis, Fucus serratus, Littorina saxatilis* and *Enteromorpha intestinalis.* The mussel *Mytilus edulis* was selected because it has been widely studied as an indicator of many metals (see Cossa, 1989). *Fucus serratus* was also chosen on a similar basis, for dissolved metal levels. *M. edulis* and *F. serratus* were collected from approximately the same tidal level. Similarly, *Littorina saxatilis* and *Enteromorpha intestinalis* were collected from the upper shore. Metal levels in *L. saxatilis* were

Table 3.1 Some useful biomonitors of metals and metalloids. After Bryan *et al*. (1985)

Metal	Indicators of dissolved metals (direct or via diet)	Indicators of dissolved and particulate metals	Indicators of sediment metals (direct or via diet)
Ag	*Littorina saxatilis*	*Cerastoderma edule*	*Scrobicularia plana* *Macoma balthica*
As	*Fucus vesiculosus* *Littorina littoralis (obtusata)*	*Cerastoderma edule*	*Scrobicularia plana* *Macoma balthica*
Cd	*Littorina littoralis (obtusata)* *Patella vulgata* *Nucella lapillus* *Fucus vesiculosus* *Ascophyllum nodosum*	*Mytilus edulis*	*Scrobicularia plana*
Co	*Fucus vesiculosus*	*Mytilus edulis*	*Nereis diversicolor*
Cr	*Ascophyllum nodosum*	*Mytilus edulis*	*Scrobicularia plana*
Cu	*Fucus vesiculosus* *Ascophyllum nodosum* *Patella vulgata* *Nucella lapillus*	*Ostrea edulis*	*Nereis diversicolor* *Nephthys hombergi*
Hg	*Fucus vesiculosus*	*Mytilus edulis*	*Scrobicularia plana* *Macoma balthica* *Platichthys flesus*
Ni	*Fucus vesiculosus*	*Cerastoderma edule*	*Scrobicularia plana*
Pb	*Ascophyllum nodosum*	*Mytilus edulis*	*Scrobicularia plana*
Se	*Fucus vesiculosus*	*Mytilus edulis*	*Scrobicularia plana* *Macoma balthica*
Sn	*Fucus vesiculosus*	*Mytilus edulis*	*Scrobicularia plana*
Zn	*Fucus vesiculosus* *Ascophyllum nodosum*	*Ostrea edulis*	*Scrobicularia plana*

examined because it is the species of interest for metal tolerance studies and to examine how tolerance could affect its potential as a biomonitor. *E. intestinalis* was chosen to give an indication of dissolved metal availability at *L. saxatilis* shore level as well as in likely algal food.

3.2 MATERIALS AND METHODS

Five sites were characterized for heavy metals: four estuaries (Castletown, Laxey, Peel and Ramsey) and a coastal site (Derbyhaven) (see chapter 2, Fig. 2.1). Sediment studies were carried out only in the estuarine sites. *Mytilus edulis* was also not sampled at Derbyhaven; and, was only available in limited numbers at the Castletown Estuary.

3.2.1 Sediment

Sediment samples were collected from the Castletown, Laxey Peel and Ramsey estuaries. Samples were obtained from three points along each estuary (mouth, middle and upper) from the oxidised surface layer (in the upper estuaries, the oxidised layer was so thin that some reduced sediment may have inevitably been included despite efforts not to do so) and placed in sealed plastic bags. Collection was made by scraping with an acid washed plastic spade. Two sets of five replicate samples were collected from each location in June 1995 and frozen until analysed.

Metal concentration in sediment is affected by particle size (Ackerman, 1980) and organic content (Bernard, 1995). Sediment particle size analysis and estimation of organic matter were therefore made. For particle size analysis, thawed sediment samples were oven-dried at 60 ° C and disaggregated before shaking through a range of sieves using a mechanical shaker (Endecotts Octagon 200).The range of sieves used

conformed to the Wentworth scale (Buchanan, 1984). The median particle size for each sample (Mdϕ) was estimated by graphical interpolation from a cumulative frequency curve. The inclusive graphic standard deviation (σ_I) and inclusive graphic skewness (SK_I) were also calculated using the formulae:

$$\sigma_I = (\phi 84 - \phi 16)/4 + (\phi 95 - \phi 5)/6.6$$

$$SK_I = [(\phi 16 + \phi 84 - 2\phi 50)/ 2(\phi 84 - \phi 16)] + [(\phi 5 + \phi 95 - 2\phi 50)/ 2(\phi 95 - \phi 5)]$$

The values obtained were checked against classification tables in Buchanan (1984).

A second set of samples were similarly oven dried and passed through the <500μ sieve for the estimation of organic matter and for metal analysis. Sediment organic matter was estimated by ashing 10 (± 0.01) g of dried sediment (<500μ) in a muffle furnace at 400 ° C for 6 hrs (Bryan *et al.*, 1985). The difference in weight expressed as percentage loss on ignition gives a good indication of percentage sediment organic matter (Buchanan, 1984).

The fraction of a sediment sample and the extraction method employed would influence the concentration of metals obtained. Much of the metals are held in the <63μ fraction (Warren, 1981, Guerzoni, *et al.*, 1984) and this size band is considered suitable for metal analysis. Some authors have suggested that it is only by examining the pelitic fraction (<2μm) of sediment that the degree of anthropogenic contamination of sediment can be properly estimated (Förstner, 1977; Helmke *et al.*, 1977). Contaminant metal may reach the sediments in fairly coarse particulate form especially from mining and smelting wastes where relatively more metal have been associated

with coarser size fractions than the finer size fractions (Jennett & Foil, 1979; Dossis & Warren, 1980). There appears to be no general agreement as to which fraction of sediment should be used for heavy metal determinations (Grant & Middleton, 1990). Indeed, various other fractions ; <100μ (Bryan *et al.*, 1985; Bernard, 1995), <500μ (Southgate *et al.*, 1983), or sometimes bulk sediment (Bryan & Gibbs, 1983, Jones, 1995), have also been used. In this work the <500μ was used to enable comparison with Southgate *et al.* (1983).

Several methods can be used to extract metals from sediment (reviewed by Förstner & Wittman, 1979; Langston & Spence, 1994). Extraction with concentrated nitric acid (Bryan *et al.*, 1985) or 4:1 nitric:hydrochloric acid mixture (Southgate *et al.*, 1983) are suitable for the determination of "total" sediment metal. I used 4:1 nitric:hydrochloric acid mixture for the extraction of total metals in sediment. 1.00 g of each sample was placed in a 50 ml conical flask and extracted at 50 ° C in 20 ml of acid (analaR grade, BDH). Five replicates of sediment were analysed from each location and three replicates of reference material of estuarine sediment (European Communities Bureau of Reference-BCR ref. material 227) (see Appendix A1) and blanks were included. The residues were washed off with 0.1 N nitric acid, filtered through acid-cleaned membrane filters and made up to a known volume before analysis. Analysis was performed on a Pye Unicam SP9 Atomic Absorption Spectrophotometer (air-acetylene flame mode) calibrated with standards of known concentrations. The extracts, references and blanks were analysed for Zn, Pb, Cu, Cd and Fe.

3.2.2 Biomonitors

The selected invertebrate and seaweed species were collected during low tide from the respective sites . After collection the seaweed were washed in double distilled water and frozen until processed for analysis. For *M. edulis* the shells were scraped free of fouling organisms before the animals were washed and placed in filtered seawater to defecate for 24 hrs (Bordin *et al.*, 1992; Mitra & Choudhury, 1993; Usero *et al.*, 1996). *L. saxatilis* were also placed in filtered seawater to defecate for 24 hrs. The water was changed every 6 hrs since no aeration was provided. Subsequently, *M. edulis* was frozen straightaway but *L. saxatilis* was first boiled for a few seconds to facilitate extraction from the shell.

The size of fucoids and the portion of the thalli used can affect the concentration of metals obtained (Bryan & Hummerstone, 1973a; Melhuus *et al.*, 1978, Kangas & Autio, 1986). To control for such variation a standardized size (~40-50 cm) was chosen. Also, sections were taken ca. 20 cm from the holdfast and only the mid sections of the thalli were included. No standardization was made for *E. intestinalis* because, being ephemeral, it was assumed that the clumps sampled were of approximately the same age. In invertebrates as well, size is an important determinant of metal levels. The relationship between size of molluscs and metal concentration is variable and depends on the metal and species (Boyden, 1977; Thompson *et al.*, 1984;

Amiard *et al.*, 1986; Bordin *et al.*, 1992; Usero, *et al.*, 1996). In this study, *M. edulis* of 45 ± 5mm shell length and *L. saxatilis* of 10 ± 2mm shell height were selected.

After thawing, *M. edulis* and *L. saxatilis* tissues were extracted from their shells. For *M. edulis*, each replicate was obtained from five pooled individuals, while ten individuals were pooled for each *L. saxatilis* replicate. Appropriate sections were also obtained from *F. serratus* with sections from each frond accounting for a replicate. Five replicates were used in each case. The wet weights of the tissues were taken and then oven-dried to constant weight at 105 ° C. *M. edulis*, *F. serratus* and *E. intestinalis* were ground in porcelain mortar and 0.5 (± 0.001) g of ground tissue measured for digestion. *L. saxatilis* tissues were digested without further maceration. Digestion was made in analaR grade nitric acid according to Harper *et al.* (1989). Blanks and reference materials of *Ulva lactuca* (European Communities Bureau of Reference-BCR ref. material 279) and *Mytilus edulis* (European Communities Bureau of Reference-BCR ref. material 278) were included in the batches of seaweed and mollusc digests respectively (see Appendix A2). Digests were filtered in acid-cleaned membrane filters, made up to a known volume in 0.1 N nitric acid and analysed by atomic absorption spectrophotometry (as described above) for Zn, Pb, Cu and Cd.

3.3 RESULTS

3.3.1 Sediment analysis

Sediment particle size and organic content

The median grain size values (Mdϕ) indicate that the sediment in upper estuary is very fine sand in Ramsey, fine sand in Castletown and Laxey, and medium sand in Peel. In general, no clear gradient was found in Mdϕ along any estuary (Table 3.2a). The contributions of various grain fractions to the Mdϕ are given by the graphic standard deviation and graphic skewness (see Table 3.2b). The sediments from most sites were not surprisingly poorly sorted, but most were also coarse skewed indicating that larger particles were important components.

The Mdϕ does not have a significant bearing on sediment metal burdens. The silt-clay (<63μ) fraction is known to contain much of the metal in sediment. Since the <500μ fraction was used in the metal analysis, the percentage of <63μ particles in that fraction was calculated for each location (see Fig. 3.1). Mean proportion of particles <63μ ranged from <1% at the mouth of Ramsey and Laxey estuaries to nearly 50 % in upper Ramsey. A clear gradient was observed in each estuary (except Peel) with higher values at the upper reaches and a seaward reduction. At Peel, the gradient was distorted by a higher mean value in the middle.

The organic content of sediments (% loss-on-ignition) is presented in Fig. 3.2. In all estuaries, the highest values were obtained in the upper reaches with a consistent decrease towards the mouth. In the upper estuary, Ramsey had the highest organic

Table 3.2a Median sediment particle size (Mdϕ) at different points along estuarine harbours in the Isle of Man.

	upper	middle	mouth
Castletown	2.6	1.94	2.56
Laxey	2.06	1.16	1.82
Peel	1.48	2.4	2.54
Ramsey	3.42	1.96	2.4

Table 3.2b Classification of sediment on the basis of graphic standard (σ_I) deviation and graphic skewness (Sk_I).

	upper	middle	mouth
Castletown	Very poorly sorted Strongly coarse skewed	Poorly sorted Coarse skewed	Moderately sorted symmetrical
Laxey	Poorly sorted Coarse skewed	Poorly sorted Strongly coarse skewed	Moderately sorted symmetrical
Peel	Very poorly sorted Coarse skewed	Poorly sorted Symmetrical	Poorly sorted Strongly coarse skewed
Ramsey	Poorly sorted Strongly coarse skewed	Poorly sorted Strongly coarse skewed	Well sorted Symmetrical

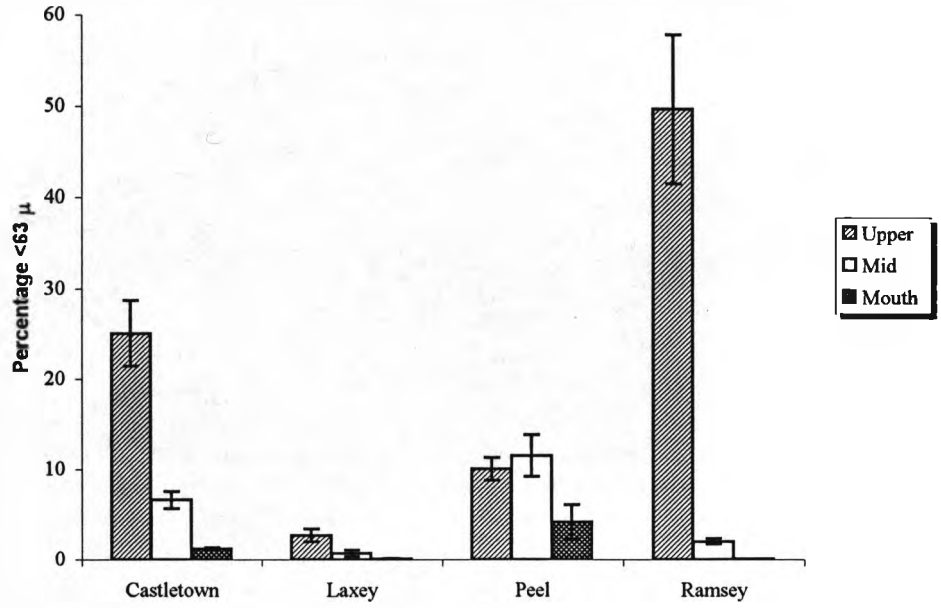

Fig 3.1 Proportion of particles < 63 μ (in the < 500μ fraction) in sediment from estuarine habours in the Isle of Man. Values are mean ± s.d., n=5. Upper, mid and mouth refer to locations along each estuary.

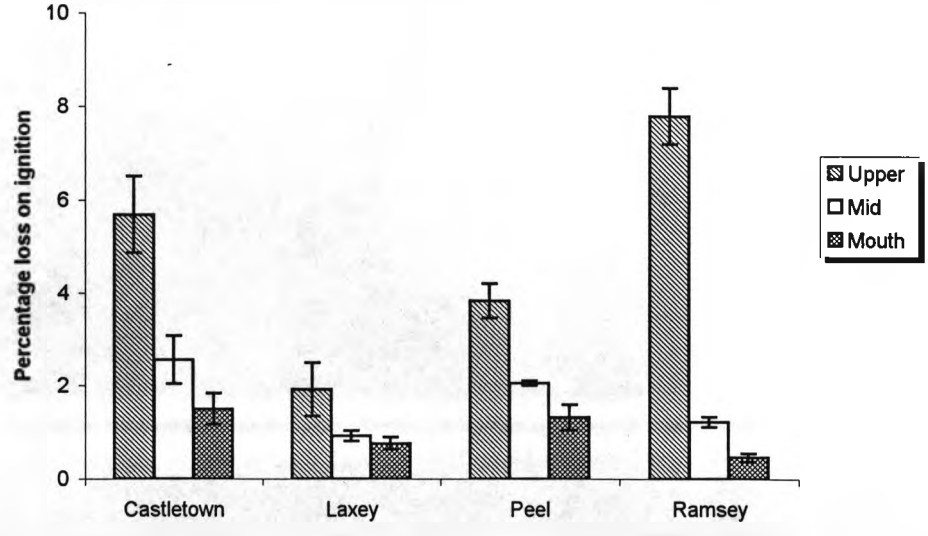

Fig. 3.2 Mean content of organic matter (± s.d., n=5) in sediment from four estuarine harbours in the Isle of Man. Upper, mid and mouth refer to locations along each estuary.

content (7.8 ± 0.6 %) and Laxey the lowest (1.5 ± 0.6 %) but the highest at the mouth of the estuary was recorded in Castletown (1.5 ± 0.3 %). There were significant positive correlations between organic content and proportion of particles <63μ in each estuary (Table 3.3); the only exception being Peel where no significant correlation was found. A significant correlation was also obtained with all sites pooled ($n=12$, $r=0.957$, $p<0.001$).

Heavy metals in sediment

Comparisons of the concentrations of Zn, Pb, Cu, Cd and Fe in sediments from different estuaries and between different locations along each estuary are given in Fig 3.3. With the exception of Cu in Laxey and Cd in Castletown, all metals showed a concentration gradient within each estuary. The highest values were obtained in the upper reaches, with a seaward reduction culminating in the lowest values at the mouth.

The highest concentrations of all metals were recorded in Peel (Fig. 3.3 , Table 3.3a). For Zn, both Peel and Laxey showed much higher values than Castletown and Ramsey; Peel being higher by a factor of about 30 and Laxey by a factor of 5 (Table 3.3b). Pb was also much higher at Peel (by a factor of 30) than Castletown and Ramsey. At Laxey, Pb was not as high as expected, exceeding values in Ramsey and Castletown only by a factor less than 2. The levels of Cu, Cd and Fe were also generally higher in Peel than at all other sites. Fe was higher in the upper estuary at Ramsey than at Laxey but similar levels of Cu were obtained.

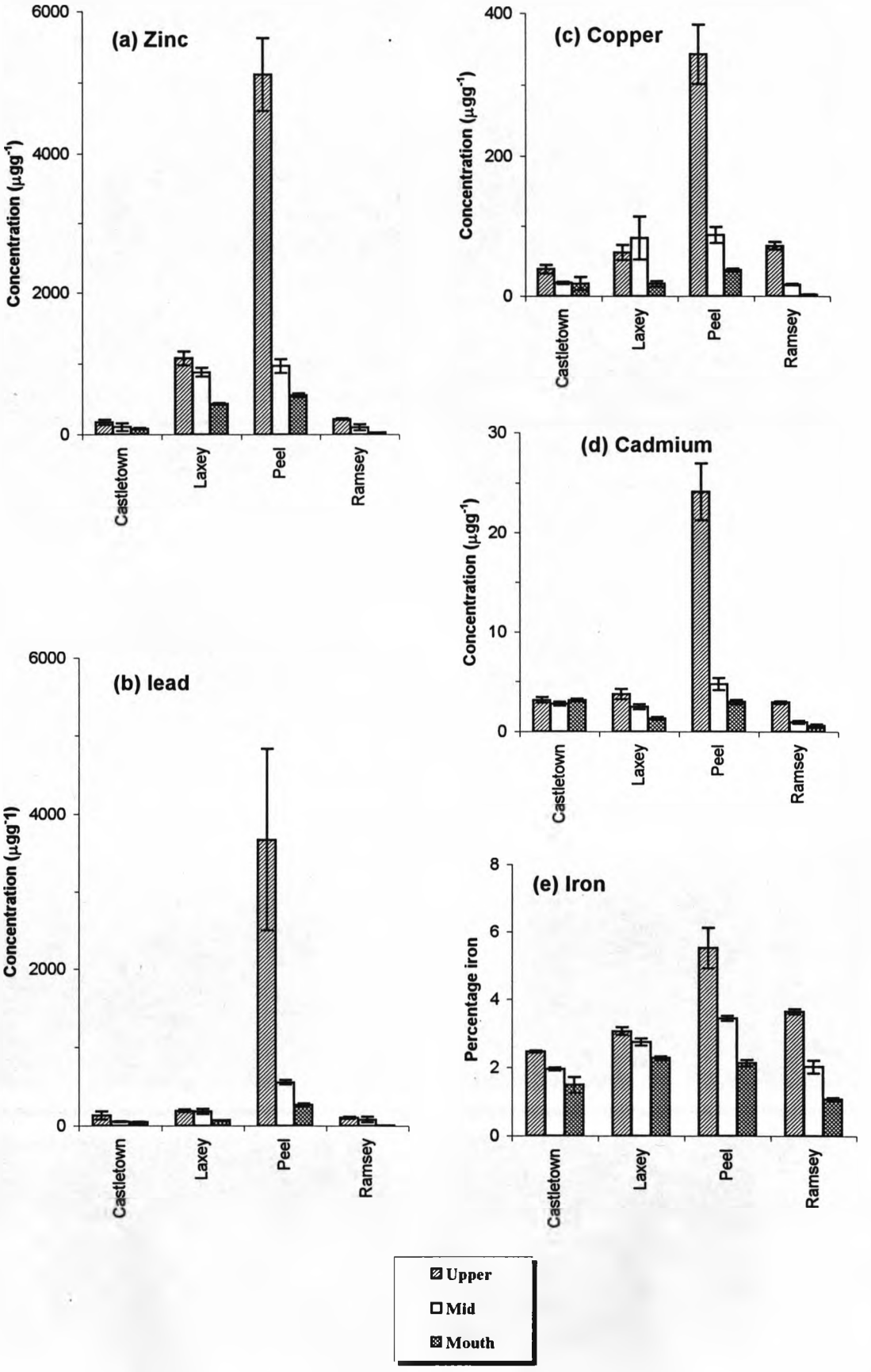

Fig. 3.3 Concentrations (mean ± s.d., n=5) of zinc, lead, copper, cadmium and iron in sediment from four estuaries in the Isle of Man. Upper, mid and mouth refer to locations in each estuary. Concentrations are expressed as dry weight of sediment (<500µ).

Table 3.3 Highest mean concentrations of metals in sediment from estuaries around the Isle of Man (upper estuary values, except copper in Laxey = mid estuary) and the ratios of metals between different sites.

(a) Concentraton of metals ($\mu g\ g^{-1}$ dry) weight except Fe (%)

	Zinc	Lead	Copper	Cadmium	Iron
Peel	5117	3668	342.9	24.12	5.52
Laxey	1075	190	82.8	3.75	3.06
Castletown	175	129	38.5	3.16	2.46
Ramsey	219	105	71.8	2.92	3.64

(b) Ratios of metals between sites

Numerator -> Denominator		Peel	Laxey	Castletown	Ramsey
Peel	---				

Laxey					
	Zinc	4.8			
	Lead	19.3			
	Copper	4.1			
	Cadmium	6.4			
	Iron	1.8			
Castletown					
	Zinc	29.3	6.2		
	Lead	28.5	1.5		
	Copper	8.9	2.2		
	Cadmium	7.6	1.2		
	Iron	2.2	1.2		
Ramsey					
	Zinc	23.4	4.9	0.8	
	Lead	35.0	1.8	1.2	
	Copper	4.8	1.2	0.5	
	Cadmium	8.3	1.3	1.1	
	Iron	1.5	0.8	0.7	---

Table 3.4 presents correlations between metals, sediment organic content and percentage of fine particles in sediment (<63μ).With a few exceptions all metals were significantly correlated with Fe in all estuaries ($p<0.001$). Pb in Castletown ($p<0.01$) and Cu in Laxey ($p<0.05$) also showed significant correlation with Fe, but Cd in Castletown was not significant. There were highly significant correlations ($p<0.001$) between sediment organic content and all metals in Peel (Table 3.4). Significant correlations also occurred between organic content and all metals in Castletown, Laxey and Ramsey except for Cu in Laxey and Cd in Castletown. Apart from Fe, no metal was significantly correlated with % <63μ fraction at Peel but at Ramsey all metals showed high correlations with that fraction. Cd in Castletown and Cu in Laxey also showed no significant correlation with the % <63μ fraction. When values for all estuaries were pooled for the correlations, all metal-metal correlations were significant ($p<0.001$, see Table 3.4). Correlations with proportion of sediment < 63μ were only significant with sediment organic content. The only metals that showed significant correlations with sediment organic content were Fe and Cd.

3.3.2 Biomonitors

Mytilus edulis* and *Fucus serratus

Zn levels in *Mytilus edulis* measured in April, 1994 in all four estuaries reflected a similar pattern with levels in sediment with the animals from Peel and Laxey showing more contamination than those from Castletown and Ramsey (Fig 3.4a). Analysis of Variance (ANOVA) showed a significant difference between sites and Tukey HSD

Table 3.4 Product moment correlation coefficients; metal-metal correlations in sediment and between metals and sediment organic content (% o.m.) and proportion of sediment <63 μ. Values are positively correlated unless otherwise indicated.
* n=3; ** n=12 for correlations with % <63μ.

	% <63 μ	% o.m.	Iron	Zinc	Lead	Copper	
Castletown							
% o.m.	**0.999**						
Iron	0.958	**0.867**					n=15*
Zinc	**0.999**	0.693	**0.738**				
Lead	**0.992**	0.720	0.704	0.621			
Copper	**0.988**	**0.821**	**0.755**	0.555	0.701		
Cadmium	0.375	0.185	0.038	0.347	0.056	0.247	
Laxey							
% o.m.	**0.996**						
Iron	0.915	**0.785**					n=15*
Zinc	0.864	**0.702**	**0.933**				
Lead	0.722	0.599	**0.896**	**0.900**			
Copper	0.411	0.314	0.605	**0.724**	**0.841**		
Cadmium	**0.961**	**0.812**	**0.914**	**0.965**	**0.802**	0.596	
Peel							
% o.m.	0.585						
Iron	0.665	**0.952**					n=15*
Zinc	0.405	**0.961**	**0.936**				
Lead	0.400	**0.880**	**0.938**	**0.947**			
Copper	0.469	**0.973**	**0.937**	**0.982**	**0.904**		
Cadmium	0.400	**0.957**	**0.933**	**0.999**	**0.948**	**0.981**	
Ramsey							
% o.m.	**0.998**						
Iron	0.943	**0.950**					n=15*
Zinc	0.941	**0.911**	**0.981**				
Lead	0.709	**0.659**	**0.842**	**0.870**			
Copper	**0.986**	**0.980**	**0.976**	**0.940**	0.745		
Cadmium	**0.994**	**0.984**	**0.964**	**0.935**	0.706	**0.987**	
All Sites Pooled							
%o.m.	**0.957**						
iron	0.393	**0.480**					n=60**
Zinc	-0.034	0.130	**0.841**				
lead	0.021	0.173	**0.809**	**0.948**			
Copper	0.067	0.246	**0.886**	**0.966**	**0.910**		
cadmium	0.136	0.283	**0.822**	**0.975**	**0.958**	**0.960**	

Underlined	p<0.05
Bold	p<0.01
Bold + Underline	p<0.001
others	p>0.05

multiple comparisons indicated that animals from Peel and Laxey had significantly higher Zn values than those from the other sites (Table 3.5a). The concentrations of Zn measured in June, 1995 (Castletown excluded) (Fig 3.4b) showed that mussels in the mid reaches of the estuary accumulated more Zn than those at the mouth. For the samples from Peel, there was a significant within-site variation in Zn levels ($p<0.001$) but significant differences did not occur with location for mussels from Laxey or Ramsey (Table 3.5b).

The concentrations of Cu in *Mytilus* measured in April 1994 and June 1995 showed variable contamination levels (Fig 3.4c & d). In the April samples, the highest mean values were obtained in animals from Laxey and ANOVA did not show any significant difference in Cu levels between sites (Table 3.6a). However, the highest mean Cu values in the June samples were obtained in the mussels from Peel and ANOVA detected a significant difference between sites (Table 3.6b). Tukey tests showed significantly higher Cu levels in animals from Peel ($p<0.001$) but no significant differences occurred in concentration between samples from Laxey and Ramsey. Also, no significant difference in Cu levels occurred between locations in any estuary.

Pb burdens in *M. edulis* were also higher at Peel than Laxey but the mean Cd value was higher in mussels from Laxey (Fig 3.4e & f). Both Laxey and Peel had significantly higher ($p<0.001$) concentrations of Pb in *Mytilus* in comparison with Ramsey but no significant differences were found between locations in any estuary (Table 3.7). *M. edulis* from both Laxey and Peel showed significantly higher values ($p<0.001$) of Cd than mussels from Ramsey (Table 3.8). In Laxey, the concentration of Cd in the

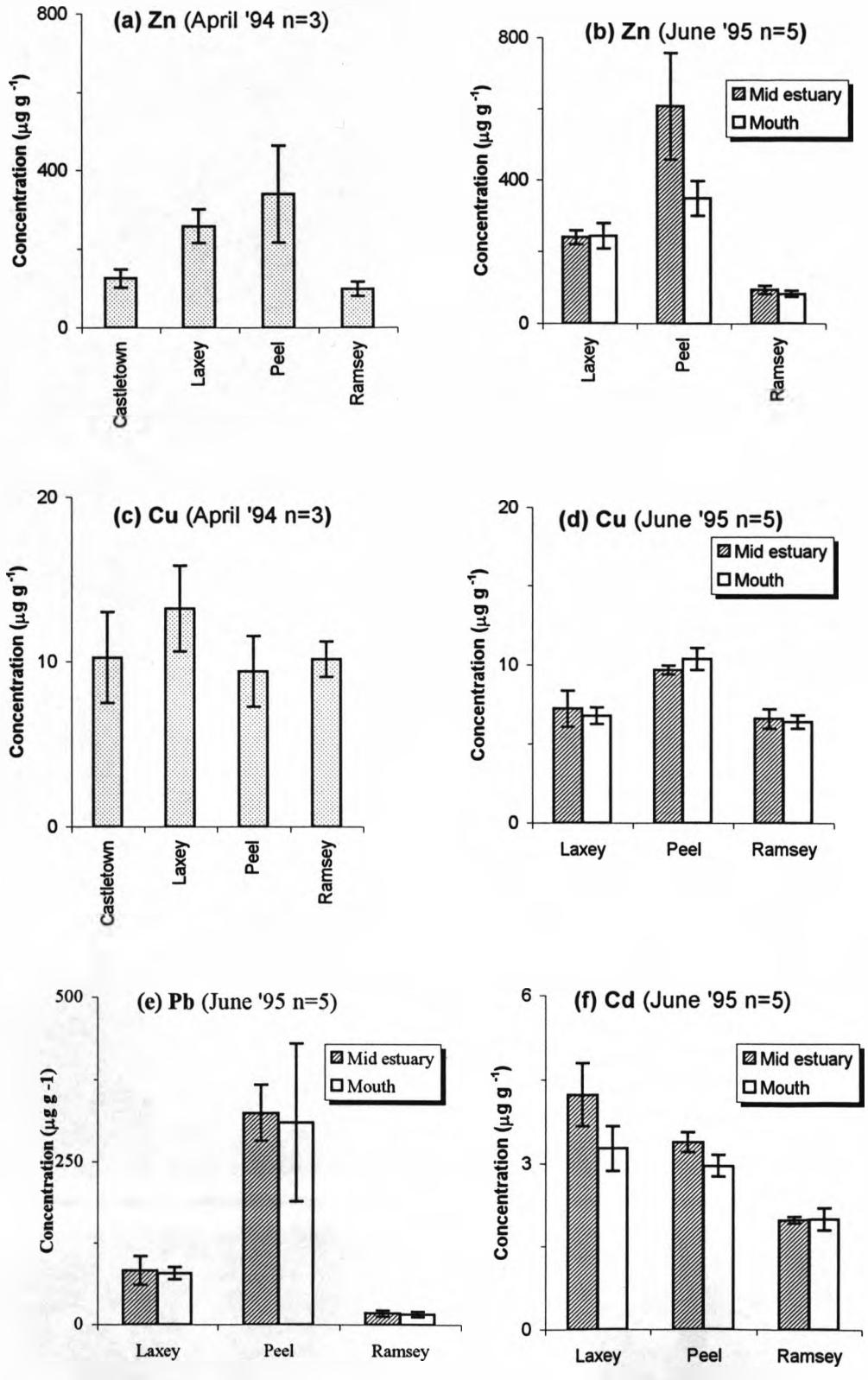

Fig. 3.4 Concentrations ($\mu g g^{-1}$ dry weight) of Zn, Pb, Cu and Cd in *Mytilus edulis* from estuaries around the Isle of Man measured in May 1994 (four estuaries) and June 1995 (Two locations in each of three estuaries). Values are mean $\pm$ s.d., n as indicated.

Table 3.5 One-way Analysis of Variance on concentrations ($\mu g\ g^{-1}$ dry weight) of zinc [log(x+1) transformed] in *Mytilus edulis*. (a) samples from four estuaries in April 1994; (b) samples from three estuaries (two locations per estuary) in May 1995.

(a) ANOVA on April 1994 samples (includes animals from Castletown)

Source of Variation	df	SS	MS	F	P-value
Site	3	0.5798	0.1933	20.871	<0.0001
Error	8	0.0741	0.0093		
Total	11	0.6539			

Significance levels for Tukey HSD comparisons on mean Zn levels (April 1994)

	Peel	Laxey	Castletown
Laxey	ns		
Castletown	**	*	
Ramsey	***	**	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

(b) ANOVA on May, 1995 samples (no samples from Castletown)

Source of Variation	df	SS	MS	F	P-value
Site	5	2.725	0.545	125.911	<0.0001
Error	24	0.104	0.004		
Total	29	2.829			

Significance levels for Tukey HSD comparisons on mean Zn levels(May 1995)

	Peel-mid	Peel-Mouth	Laxey-mouth	Laxey-mid	Ramsey-mid
Peel-Mouth	***				
Laxey-mouth	***	*			
Laxey-mid	***	**	ns		
Ramsey-mid	***	***	***	***	
Ramsey-mouth	***	***	***	***	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.6 One-way Analysis of Variance (ANOVA) on concentrations (μg g^{-1} dry weight) of copper [log(x+1) transformed] in *Mytilus edulis* from estuaries in the Isle of Man.

(a) ANOVA on April 1994 values, includes samples from Castletown estuary

Source of Variation	df	SS	MS	F	P-value
Site	3	0.0364	0.0121	1.911	0.206
Error	8	0.0508	0.0064		
Total	11	0.0873			

(b) ANOVA on June 1995 samples from three estuaries (two locations per estuary) No values from Castletown estuary

Source of Variation	df	SS	MS	F	P-value
Site	5	0.1674	0.0335	27.934	<0.0001
Error	24	0.0288	0.0012		
Total	29	0.1962			

Significance levels for Tukey HSD comparisons on mean copper levels

	Peel-Mouth	Peel-mid	Laxey-mid	Laxey-mouth	Ramsey-mid
Peel-mid	ns				
Laxey-mid	***	***			
Laxey-mouth	***	***	ns		
Ramsey-mid	***	***	ns	ns	
Ramsey-mouth	***	***	ns	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.7 One-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of lead [log(x+1) transformed] in *Mytilus edulis* from three estuaries (two locations per estuary) in the Isle of Man.

Source of Variation	df	SS	MS	F	P-value
Site	5	7.764	1.553	164.344	<0.0001
Error	24	0.227	0.009		
Total	29	7.990			

Significance levels for Tukey HSD comparisons on mean lead levels

	Peel-mid	Peel-Mouth	Laxey-mid	Laxey-mouth	Ramsey-mouth
Peel-Mouth	ns				
Laxey-mid	***	***			
Laxey-mouth	***	***	ns		
Ramsey-mouth	***	***	***	***	
Ramsey-mid	***	***	***	***	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.8 One-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of cadmium [log(x+1) transformed] in *Mytilus edulis* from three estuaries (two locations per estuary) in the Isle of Man.

Source of Variation	df	SS	MS	F	P-value
Site	5	0.2342	0.0468	51.615	<0.0001
Error	24	0.0218	0.0009		
Total	29	0.2560			

Significance levels for Tukey HSD comparisons on mean cadmium levels

	Laxey-mid	Peel-mid	Laxey-mouth	Peel-mouth	Ramsey-mouth
Peel-mid	**				
Laxey-mouth	***	ns			
Peel-mouth	***	ns	ns		
Ramsey-mouth	***	***	***	***	
Ramsey-mid	***	***	***	***	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

animals was also significantly higher in samples from the upper reaches than at the mouth of the estuary.

The values of Cd and Pb generally showed the same pattern of contamination between *F. serratus* and *M. edulis* but Zn and Cu were different. The level of Pb in *F. serratus* was highest in Peel (Fig 3.5a) and the seaweed from both Peel and Laxey showed significantly higher mean values than those from Ramsey, Castletown and Derbyhaven ($p<0.001$, see Table 3.9). For Cd, the plants from Laxey showed the highest concentration (Fig 3.5b) and significantly higher mean values were observed only between Laxey and other sites ($p<0.001$, Table 3.10). Fig. 3.5c shows the differences in Zn concentrations in *F. serratus*. Significantly elevated Zn concentrations ($p<0.001$) were obtained in plants from both Laxey and Peel in comparison with other sites (see Table 3.11). It sould be noted that Zn values in *F. serratus* were much higher in the plants from Laxey than samples from Peel. This is contrary to the Zn levels in *M. edulis* which were higher in the animals from Peel . Cu levels in *F. serratus* were higher in Laxey and Peel than other sites (Fig 3.5d). The plants from Laxey had significantly higher Cu levels than samples from all other sites (Table 3.12). Cu contamination was also significantly higher in the seaweed from Peel than samples from Castletown and Derbyhaven but not Ramsey. No significant differences were found in the mean concentrations of Zn and Cu sampled in March and June and there was also no significant interaction between site and month of sampling (see Tables 3.11 & 3.12).

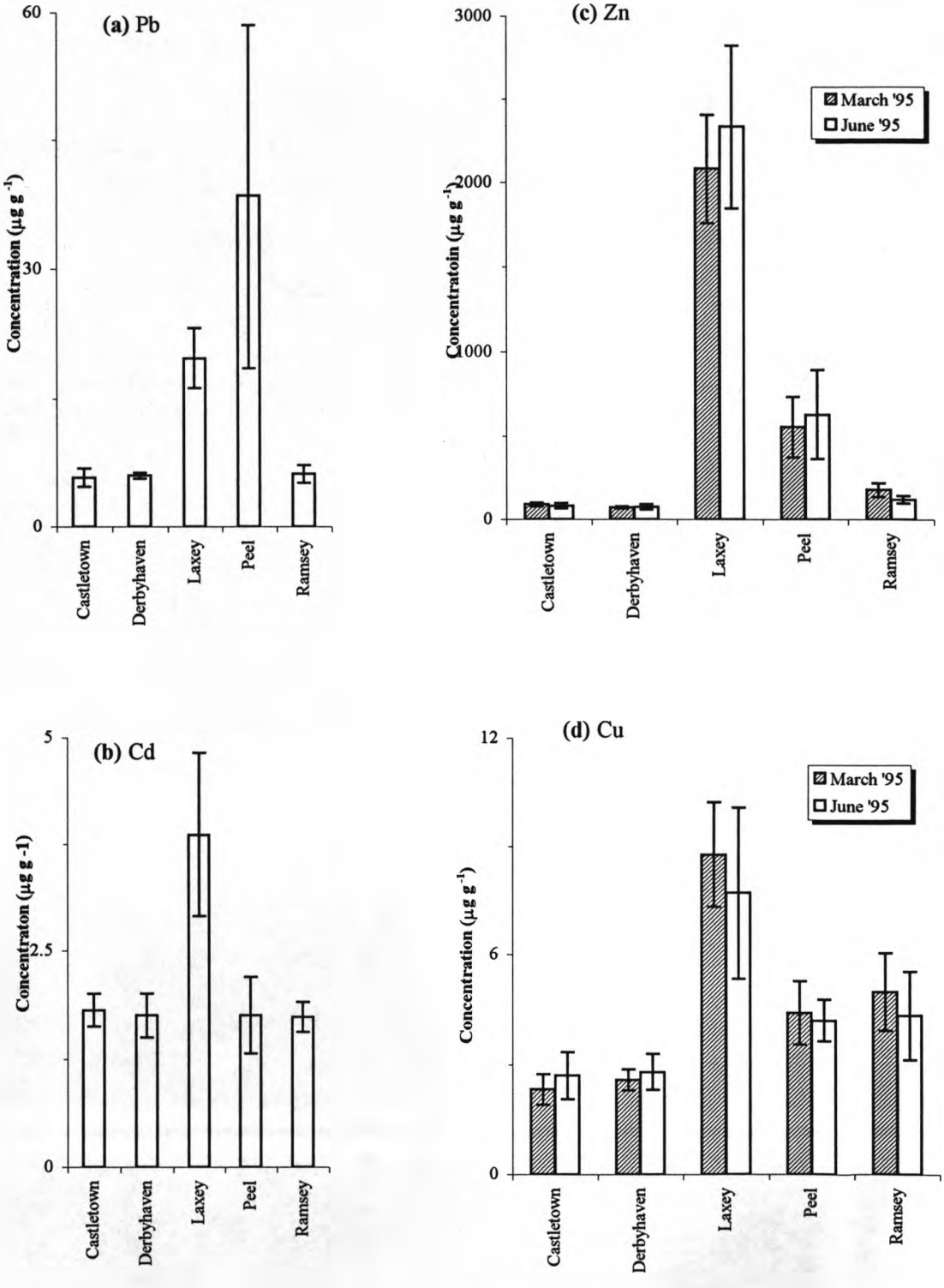

Fig. 3.5 Concentrations of zinc, lead, copper and cadmium (mean ± s.d., n=5) in *Fucus serratus* from sites around the Isle of Man. Lead and copper values are for June 1995. Concentrations are in µg g^{-1} dry weight.

Table 3.9 One-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of lead [log(x+1) transformed] in *Fucus serratus* from five sites in the Isle of Man.

Source of Variation	df	SS	MS	F	P-value
Site	4	2.2691	0.5673	54.987	<0.0001
Error	20	0.2063	0.0103		
Total	24	2.4754			

Significance levels for Tukey HSD comparisons on mean lead levels

	Peel	Laxey	Ramsey	Derbyhaven
Laxey	**			
Ramsey	***	***		
Derbyhaven	***	***	ns	
Castletown	***	***	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.10 One-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of cadmium [log(x+1) transformed] in *Fucus serratus* from five sites in the Isle of Man.

Source of Variation	df	SS	MS	F	P-value
Site	4	0.2338	0.0584	20.560	<0.0001
Error	20	0.0568	0.0028		
Total	24	0.2906			

Significance levels for Tukey HSD comparisons on mean cadmium levels

	Laxey	Castletown	Derbyhaven	Ramsey
Castletown	***			
Derbyhaven	***	ns		
Ramsey	***	ns	ns	
Peel	***	ns	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.11 Two-way Analysis of Variance on concentrations ($\mu g\ g^{-1}$ dry weight) of zinc [log(x+1) transformed] in *Fucus serratus* from five sites, sampled in March and June 1995.

Source of Variation	df	SS	MS	F	P-value
Site	4	15.6097	3.9024	357.051	<0.0001
Month	1	0.0064	0.0064	0.585	0.449
Interaction	4	0.0825	0.0206	1.888	0.131
Error	40	0.4372	0.0109		
Total	49	16.1358			

Significance levels for Tukey HSD comparisons on mean zinc levels between sites

	Laxey	Peel	Ramsey	Castletown
Peel	***			
Ramsey	***	***		
Castletown	***	***	***	
Derbyhaven	***	***	***	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.12 Two-way Analysis of Variance on concentrations ($\mu g\ g^{-1}$ dry weight) of copper [log(x+1) transformed] in *Fucus serratus* from five sites, sampled in March and June 1995.

Source of Variation	df	SS	MS	F	P-value
Site	4	1.1155	0.2789	53.749	<0.0001
Month	1	0.0016	0.0016	0.301	0.587
Interaction	4	0.0204	0.0051	0.981	0.429
Error	40	0.2075	0.0052		
Total	49	1.3450			

Significance levels for Tukey HSD comparisons on mean copper levels between sites

	Laxey	Ramsey	Peel	Derbyhaven
Ramsey	***			
Peel	***	ns		
Derbyhaven	***	***	***	
Castletown	***	***	***	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

The relationships between sediment metal levels and values in mussels were checked only for Zn as Pb and Cd values in mussels were available for only three estuaries and Cu values were inconsistent. Zn levels in *Mytilus* showed a linear relationship with values in sediment (Fig 3.6a) and the correlation was significant ($n=4$, $r=0.994$, $P<0.001$). The slope of the regression was, however, low. Significant positive correlations were also obtained between Zn values in sediment and levels in *Fucus serratus* as well as for *Mytilus/Fucus* but no regression line could be fitted (Fig. 3.6b & C). Correlations between levels of Cu and Cd in *Fucus* and values in sediment were very low but a significant correlation was obtained for Pb ($n=4$, $r=0.958$, $p<0.01$)

Littorina saxatilis* and *Enteromorpha intestinalis

Zn, Cd and Pb concentrations in *Littorina saxatilis* were generally higher in Laxey than all other sites (Fig 3.7).The mean Zn value in *L. saxatilis* from Laxey was more than double the concentrations in animals from other sites (Fig 3.7a) and ANOVA showed significant differences between sites ($p<0.0001$, Table 3.13). Animals from Laxey had significantly higher Zn levels (Tukey, $p<0.001$). The mean Zn value in animals from Peel was also significantly higher than Castletown and Derbyhaven ($p<0.001$) but not Ramsey. There was also a significant difference in Zn levels between winter (December 1994) and summer (June 1994) samples as well as a significant interaction between season and site (Table 3.13). The interaction is also reflected in the variable direction of seasonal change between sites; higher Zn values were recorded in June at Laxey and Derbyhaven whereas December values were higher at Peel, Ramsey and Castletown.

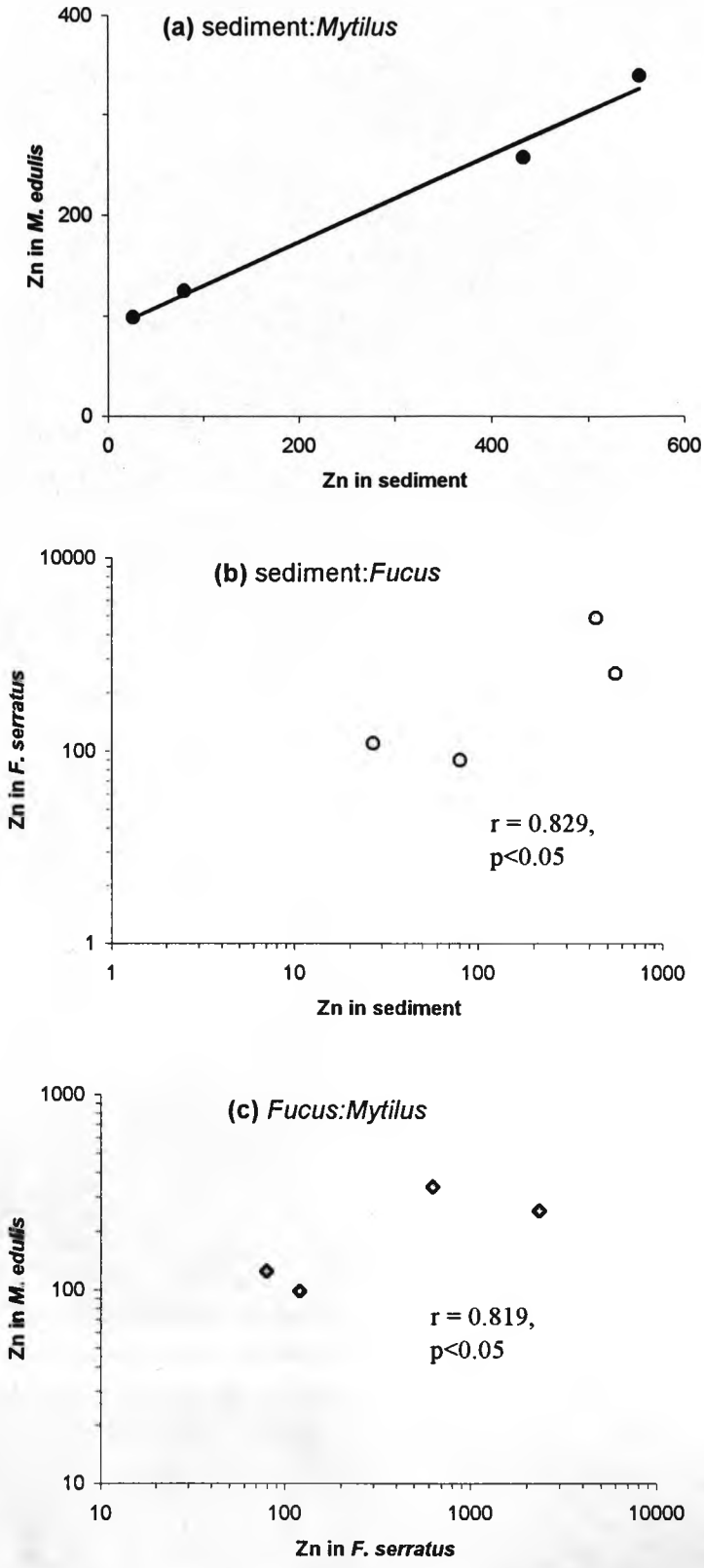

Fig. 3.6 Relationship between concentrations of zinc (μg g^{-1} dry weight) in sediment and levels in *Mytilus edulis* (a) and *Fucus serratus* (b); and, between values in *Fucus serratus* and *Mytilus edulis* (c). Regression equation for (a):

M. edulis Zn = 86.0 + 0.434 SedimentZn, $r^2 = 0.987$, $p<0.001$, n=4.

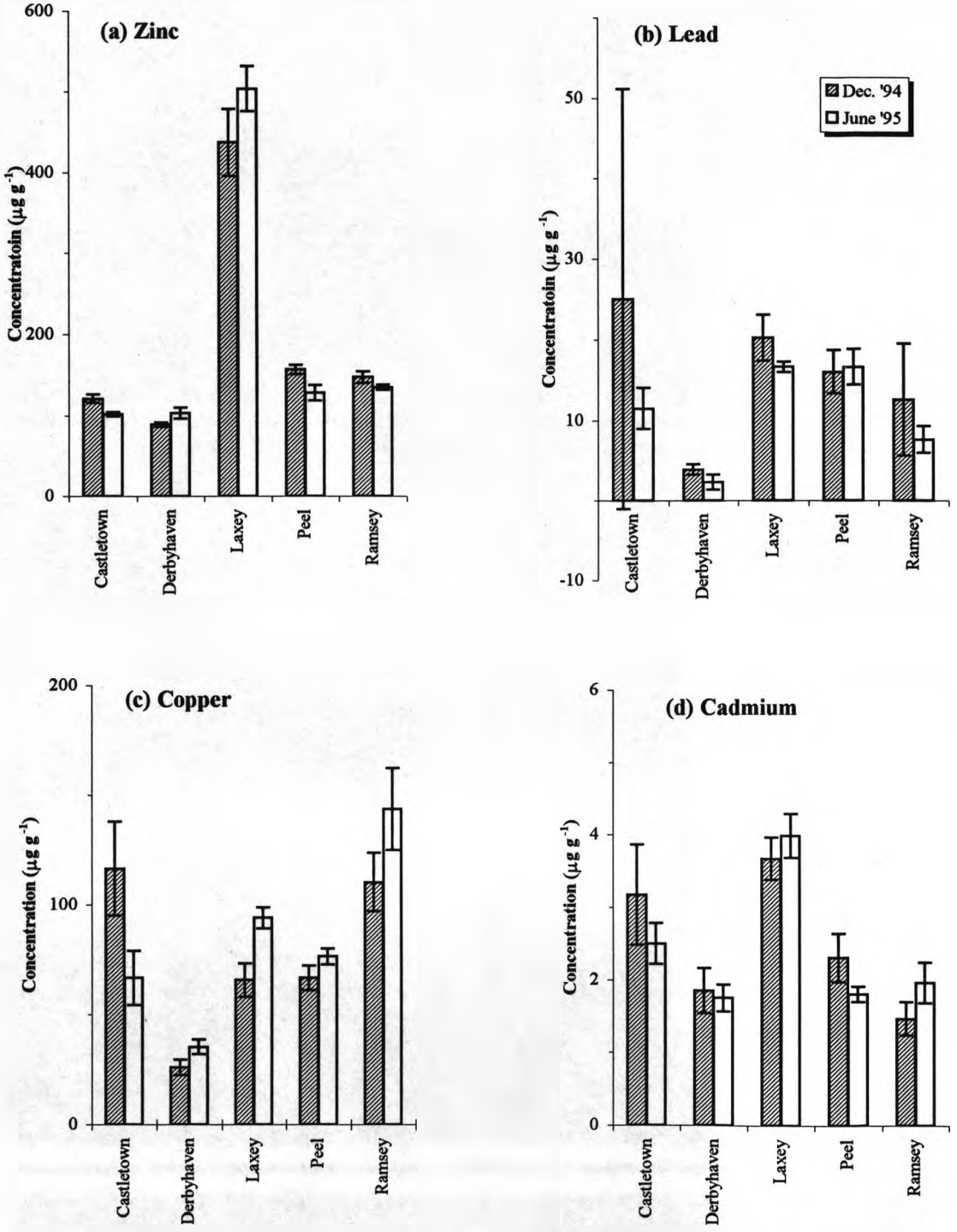

Fig 3.7 Concentrations (mean ± s.d., n=5) of zinc, lead, copper and cadmium in *Littorina saxatilis* sites around the Isle of Man. Samples collected in December 1994 (winter) and June 1995 (summer) to assess seasonal variations in metal levels.

Table 3.13 Two-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of zinc [log(x+1) transformed] in *Littorina saxatilis* from five sites, sampled in two seasons (Dec. 1994 and June 1995)

Source of Variation	df	SS	MS	F	P-value
Site	4	2.9567	0.7392	1359.768	<0.0001
Season	1	0.0027	0.0027	4.977	<0.05
Interaction	4	0.0534	0.0134	24.581	<0.0001
Error	40	0.0217	0.0005		
Total	49	3.0346			

Significance levels for Tukey HSD comparisons on mean zinc levels between sites

	Laxey	Peel	Ramsey	Castletown
Laxey				
Peel	***			
Ramsey	***	ns		
Castletown	***	***	***	
Derbyhaven	***	***	***	***

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.14 One-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of lead [log(x+1) transformed] in *Littorina saxatilis* from five sites in the Isle of Man.

Source of Variation	df	SS	MS	F	P-value
Site	4	1.8706	0.4676	69.136	<0.0001
Error	20	0.1353	0.0068		
Total	24	2.0059			

Significance levels for Tukey HSD comparisons on mean lead levels between sites

	Laxey	Peel	Castletow	Ramsey
Peel	ns			
Castletown	ns	ns		
Ramsey	***	***	*	
Derbyhaven	***	***	***	***

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Pb values in *L. saxatilis* were higher in Laxey and Peel but the magnitude of the difference between Laxey and other sites was less than it was for Zn (Fig. 3.7b). Because of some doubtful values obtained in samples from Castletown in December (see. s.d. on Fig. 3.7b), only June data were used for ANOVA which showed a significant difference between sites (Table 3.14). There was no significant difference between mean Pb in animals from Castletown and Laxey/ Peel, although the winkles from the latter sites showed significantly higher values than individuals from Derbyhaven and Ramsey (Tukey, $p<0.001$).

Cu burdens in *L. saxatilis* were higher in both Ramsey and Castletown than Laxey (Fig 3.7c) and there was a significant difference between sites (Table 3.15). The mean concentration of Cu in animals from Ramsey was significantly higher than individuals from all other sites ($p<0.001$); and, the value in Castletown was only significantly higher than Derbyhaven. There was also a significant difference in concentration with season ($p<0.01$) as well as a significant interaction between season and site ($p<0.001$). While all other sites had higher Cu levels in June, values in Castletown were higher in December.

L. saxatilis from Laxey had the highest mean value of Cd, followed by Castletown (Fig. 3.7d). Significant differences in mean Cd between sites occurred in the form (see Table 3.16):

Laxey > Castletown > Peel = Ramsey = Derbyhaven

Table 3.15 Two-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of copper [log(x+1) transformed] in *Littorina saxatilis* from five sites, sampled in two seasons (Dec. 1994 and June 1995)

Source of Variation	df	SS	MS	F	P-value
Site	4	2.0216	0.5054	149.169	<0.0001
Season	1	0.0328	0.0328	9.693	<0.001
Interaction	4	0.2725	0.0681	20.107	<0.0001
Error	40	0.1355	0.0034		
Total	49	2.4624			

Significance levels for Tukey HSD comparisons on mean copper levels between sites

	Ramsey	Castletown	Laxey	Peel
Castletown	***			
Laxey	***	ns		
Peel	***	ns	ns	
Derbyhaven	***	***	***	***

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.16 Two-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of cadmium [log(x+1) transformed] in *Littorina saxatilis* from five sites, sampled in two seasons (Dec. 1994 and June 1995)

Source of Variation	df	SS	MS	F	P-value
Site	4	0.4599	0.1150	59.336	<0.0001
Season	1	0.0030	0.0030	1.537	0.222
Interaction	4	0.0518	0.0130	6.686	<0.001
Error	40	0.0775	0.0019		
Total	49	0.5923			

Significance levels for Tukey HSD comparisons on mean lead levels between sites

	Laxey	Castletown	Peel	Derbyhaven
Castletown	***			
Peel	***	***		
Derbyhaven	***	***	ns	
Ramsey	***	***	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.17 Correlations of metal pairs in *Littorina saxatilis* from sites around the Isle of Man (n=48). All correlations are positive and values in bold are significant (p<0.001), others are not significant (p>0.05)

	Zinc	Lead	Copper	Cadmium
Zinc	---			
Lead	**0.587**	---		
Copper	0.110	0.189	---	
Cadmium	**0.836**	**0.513**	0.067	---

No significant differences in Cd values occurred between seasons but there was a significant interaction between season and site ($p<0.001$).

Correlations between metal pairs in *L. saxatilis* were highest between Zn and Cd ($n=48$, $r = 0.836$, $p<0.001$). There were also significant correlations between Zn and Pb ($n=48$, $r = 0.587$, $p<0.001$), and Pb/Cd ($n=48$, $r =0.513$, $p<0.001$). No other metal pairs showed significant correlations (Table 3.17).

The concentrations of Zn, Pb, Cu and Cd in *Enteromorpha intestinalis* are presented in Fig. 3.8. The concentrations of all metals were highest in the algae from Laxey, which also had the highest variability in concentrations. Analysis of variance showed a significant difference in concentration of all metals with site (Tables 3.18-3.21). Tukey HSD multiple comparisons showed that the plants from Laxey had significantly higher levels of Zn than those from all other sites (Table 3.18). *Enteromorpha* from Peel also had significantly higher values of Zn than the plants from Derbyhaven but not Ramsey or Castletown. Tukey tests also detected significantly higher Pb values in plants from Laxey and Peel in comparison with samples from the three other sites (Table 3.19). For Cu and Cd, the only significant differences obtained involved comparisons between mean values in Laxey samples with other sites (see Tables 3.20 and 3.21).

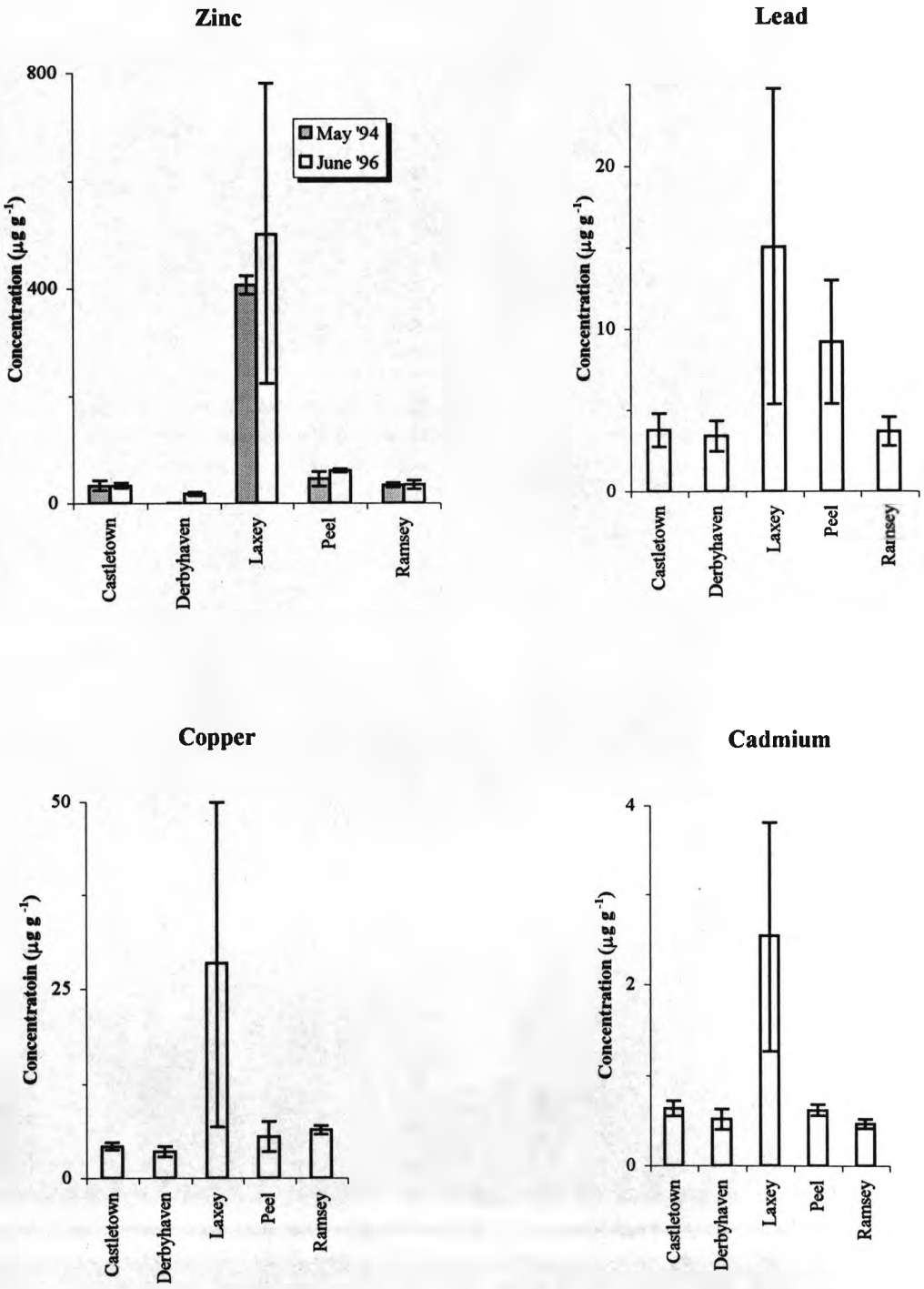

Fig. 3.8 Concentrations (mean ± s.d., n=5) of zinc, lead, copper and cadmium in *Enteromorpha intestinalis* from sites around the Isle of Man. Values for lead, copper and cadmium are for June 1996.

Table 3.18 One-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of zinc [log(x+1) transformed] in *Enteromorpha intestinalis* from five sites in the Isle of Man.

Source of Variation	df	SS	MS	F	P-value
Site	4	5.4485	1.3621	54.488	<0.0001
Error	20	0.5000	0.0250		
Total	24	5.9485			

Significance levels for Tukey HSD comparisons on mean zinc levels between sites

	Laxey	Peel	Ramsey	Castletown
Laxey				
Peel	***			
Ramsey	***	ns		
Castletwon	***	ns	ns	
Derbyhaven	***	***	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.19 One-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of lead [log(x+1) transformed] in *Enteromorpha intestinalis* from five sites in the Isle of Man.

Source of Variation	df	SS	MS	F	P-value
Site	4	1.0883	0.2721	12.948	<0.0001
Error	20	0.4202	0.0210		
Total	24	1.5085			

Significance levels for Tukey HSD comparisons on mean lead levels between sites

	Laxey	Peel	Castletown	Ramsey
Laxey				
Peel	***			
Castletown	***	*		
Ramsey	***	*	ns	
Derbyhaven	***	**	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.20 One-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of copper [log(x+1) transformed] in *Enteromorpha intestinalis* from five sites in the Isle of Man.

Source of Variation	df	SS	MS	F	P-value
Site	4	1.6682	0.4171	18.847	<0.0001
Error	20	0.4426	0.0221		
Total	24	2.1108			

Significance levels for Tukey HSD comparisons on mean copper levels between sites

	Laxey	Ramsey	Peel	Castletown
Ramsey	***			
Peel	***	ns		
Castletown	***	ns	ns	
Derbyhaven	***	ns	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

Table 3.21 One-way Analysis of Variance on concentrations (μg g^{-1} dry weight) of cadmium [log(x+1) transformed] in *Enteromorpha intestinalis* from five sites in the Isle of Man.

Source of Variation	df	SS	MS	F	P-value
Site	4	0.4463	0.1116	18.073	0.0001
Error	20	0.1235	0.0062		
Total	24	0.5698			

Significance levels for Tukey HSD comparisons on mean cadmium levels between sites

	Laxey	Castletown	Peel	Derbyhaven
Castletown	***			
Peel	***	ns		
Derbyhaven	***	ns	ns	
Ramsey	***	ns	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001; ns=p>0.05

The relationships between the values of Zn, Pb, Cu and Cd in *Littorina saxatilis* and *Enteromorpha intestinalis* are presented in Fig. 3.9. Significant positive correlations were obtained between Zn and Cd values in *L. saxatilis* and *E. intestinalis* but no significant correlations were obtained between the levels of Pb and Cu in the two species.

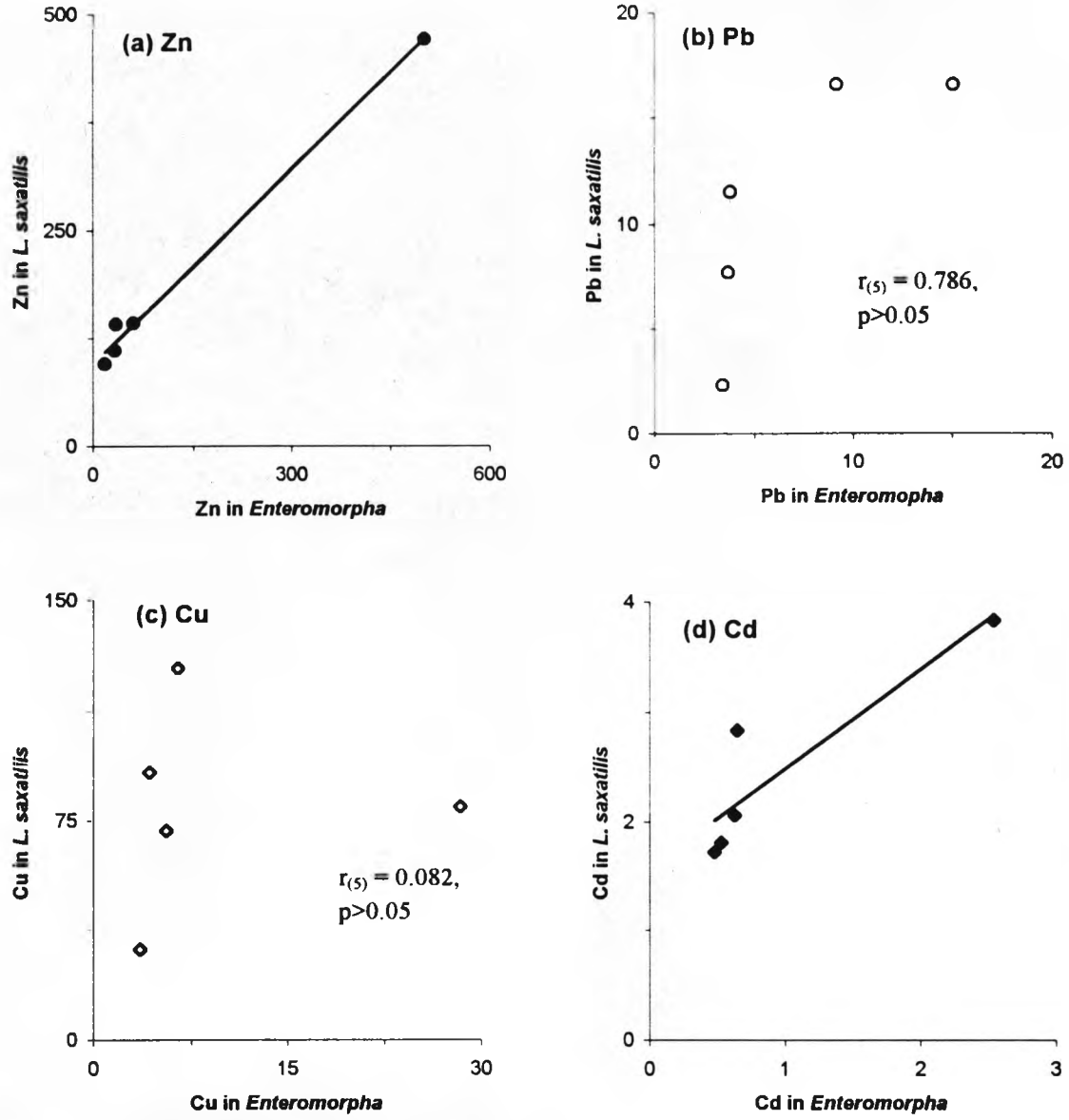

Fig. 3.9 Relationship between concentrations (μg g^{-1} dry weight) of zinc, lead, copper and cadmium in *Littorina saxatilis* and values in *Enteromorpha intestinalis*. Regression equations for Zn and Cd:

L.saxatilis Zn = 95.5 + 0.749*Enteromorpha* Zn, $r^2 = 0.993$, $p<0.001$, n=5

L.saxatilis Cd = 1.59 + 0.900*Enteromorpha* Cd, $r^2 = 0.807$, $p<0.05$, n=5.

3.4 DISCUSSION

3.4.1 Sediment metal levels

The abrupt change which takes place in estuaries as seawater mixes with freshwater results in rapid sedimentation processes (Förstner & Wittmann, 1979). Much of the suspended sediment load is derived from freshwater run-off (Duinker, 1980) but sediment is also transported into estuaries by the sea (Dyer, 1972). Clay particles tend to be deposited as a result of flocculation in seawater and the increase in salinity and sometimes pH also causes the precipitation of Fe, Al and Mn (Foster & Hunt, 1975; Bryan, 1976a; Hanson *et al.*, 1993). Other heavy metals may be removed from solution by co-precipitation with or adsorbed on these particles (Bryan, 1976a). Fe showed significant positive correlation with Zn, Pb, Cu and Cd in all estuaries, except for Cd in Castletown. This implies that co-precipitation with Fe is important in the deposition of these metals in sediment. Correlations with sediment organic content suggest that adsorption to organic matter is also a significant route of entry of all metals in sediments at Peel, Castletown, Laxey and Ramsey except for Cu in Laxey and Cd in Castletown. Luoma & Bryan (1981) similarly reported that both humic substances and Fe oxides were important in partitioning Cu, but that Zn was associated with Fe oxides in sediment from estuaries in South-west England.

Under suitable conditions, metals which have been adsorbed or incorporated into sediment particles may be desorbed or remobilised in estuaries (Bryan, 1976a, Förstner & Wittmann, 1979). In detailed studies of the Rhine Estuary, DeGroot *et al.* (1971)

showed that as sediments move downstream, metals such as Hg, Cd, Cu, Pb and Cr but not Mn are mobilised, resulting in a steady decrease in concentration. This was thought to be due to the intensive decomposition of organic matter, the product of which form soluble organometallic complexes with the consequence of a release of metals from sediments into the water. The more readily mobilised metals such as Cd and Hg were those which formed the most stable organometallic complexes. However, Müller & Förstner (1975) found that the profile of Cd, Hg and Cu in the Elbe Estuary did not conform to the mobilisation model of DeGroot *et al.* which shows that the factors controlling the distribution of trace metals vary from estuary to estuary. The re-suspension of estuarine sediment caused by tidal currents is also an important factor in the release of metals from particulates and from pore water (Lindberg *et al.*, 1975). Morris *et al.* (1982) reported from a two-year study of the Tamar Estuary that during a neap-spring tidal phase, bed stresses increased with increasing tidal currents, resulting in net re-suspension of sediment and release of sediment pore water enriched in Mn.

Contamination gradients were found for Zn, Pb and Cu (with a few exceptions, see Fig. 3.3) in all the Manx estuaries studied. It is possible that some form of desorption takes place but other factors may be more important. Parallel gradients of organic content and fine grain size distribution implies that these would have a bearing on metal concentrations in the estuaries. In Peel, the fine grain fraction was higher in the mid-estuary but metal levels were consistently higher at the upper reaches. The hydrographic conditions of Peel estuary were such that more sedimentation seemed to

occur in the middle region but the fact that metal levels were lower here than the upper reaches implies some dilution with sediment of marine origin.

Table 3.22 presents a comparison of sediment metal levels in Isle of Man estuaries with other contaminated and uncontaminated estuaries. In general, estuaries associated with present and past mining areas such as Restronguet, the Gannel, Peel and Laxey showed higher sediment levels of Zn, Pb and Cu. Estuaries in industrialised locations also showed higher contamination in sediment than control sites presumed to be clean. The highest concentrations of Zn were found in sediments from Peel and Restronguet possibly reflecting the relative differences in volume of mining; but the levels in sediment from Laxey estuary which drains the most heavily mined area was not the highest, indicating that sedimentation regimes are important (see Bryan & Gibbs, 1983, chapter 2). Similarly, Peel and the Gannel had the highest levels of Pb while Cu levels were higher in the Restronguet Creek and Dulas Bay. Pb levels reported by Southgate *et al.* (1983) for Laxey (see Table 3.23) are in the same order of magnitude as those found in the Gannel. It is worthy of note that Zn and Pb levels in the "uncontaminated" sites in the Isle of Man (Castletown and Ramsey) were much higher than those recorded for other "clean" sites (Yealm and Avon) (Bryan & Gibbs 1983).

Temporal variation in hydrological and physico-chemical factors may influence sediment metal levels. Bryan & Hummerstone (1977) found significant differences in sediment Pb in the Looe Estuary, Cornwall between December 1975 and March 1976. They suggested that seasonal changes in tidal regime and the capacity of the incoming

Table 3.22 Comparison of metal concentrations in sediment ($\mu g\ g^{-1}$ dry weight) from Isle of Man estuaries with other "contaminated" and "uncontaminated" estuaries in the United Kingdom

Site	Zinc	Lead	Copper	Source of Contamination	Reference
	Isle of Man Estuaries				
Peel	5117	3668	343	Mining	1
Laxey	1075	190	83	Mining	1
Castletown	175	129	38	"Clean"	1
Ramsey	219	105	72	"Clean"	1
	Other Estuaries				
Restronget Cr.	3515	396	2540	Mining	2
Hayle	942	218	782	Mining	2
Gannel	1215	2175	217	Mining	2
Dulas Bay	1200		2007	Mining	4
Tamar	392	156	305	Mining	2
Mersey	379	124	84	Industrial	3
Tyne	421	187	92	Industrial	3
Severn	259	89	38	Industrial	3
Yealm	110	50	35	"Clean"	2
Avon	98	39	19	"Clean"	2

1 = This study; 2 = Bryan & Gibbs, 1983; 3 = Bryan & Langston, 1992; 4 = Jones, 1995.
1 is 1:1 HNO_3:HCl digest of <500μ particles of sediment; 2 & 3 are HNO_3 digests of <100μ fraction; 4 is HNO_3 digest of bulk sediment.

Table 3.23 Comparison of Zn, Pb, Cu and Cd concentrations in sediment between values obtained in this study(a) and by Southgate *et al.* (1983) (b), for four estuarine habours in the Isle of Man.u=upper, m=middle and l/mo=lower in Southgate *et al.* and mouth in this study.

		Zinc		Lead		Copper		Cadmium	
		a	b	a	b	a	b	a	b
Peel	u	5117	7176	3668	5136	343	709	24	19
	m	965	5882	554	3173	87	361	5	10
	l/mo	554	3255	264	1356	37	466	3	7
Laxey	u	1075	2956	190	2636	62	234	4	13
	m	879	3165	181	3306	83	212	2	13
	l/mo	434	3062	70	982	18	318	1	28
Castletown	u	175	183	129	85	38	91	3	3
	m	104	155	52	103	19	96	3	2
	l/mo	80	181	44	116	18	159	3	2
Ramsey	u	219	116	105	71	72	17	3	2
	m	99	72	81	48	17	18	1	2
	l/mo	27	46	4	35	2	12	1	1

All concentrations in $\mu g\ g^{-1}$ dry sediment (<500μ) digested in 4:1 HNO_3:HCl

freshwater to carry suspended sediment may explain the difference. Such changes may be magnified over time and the concentrations of Zn, Pb, Cu and Cd in sediment obtained in this study are compared (Table 3.23) with Southgate *et al.*(1983). Their samples were collected in 1979 while those for my study were obtained in 1995, giving a 16-year difference. While similar values were obtained for Cd and Cu, considerably lower concentrations were recorded in this study for Pb and Zn, especially in the middle and lower reaches of Peel and Laxey estuaries. However, the sampling locations within each estuary may have been slightly different between the two studies. In particular, the lower reaches studied by Southgate *et al.* may not be equivalent to the mouth of the estuary in this study, assuming that the terminology in both cases conformed to McLusky's (1981) characterisation of estuaries. McLusky divided estuaries into five characteristic zones (Head, Upper, Middle, Lower and Mouth) with typical sediment types. Unfortunately, no information is available on the sediment characteristics in Southgate *et al.* (1983) for comparison.

Harbour activities such as dredging have been shown to cause remobilisation of sediment metal (Bella & McCauley, 1972; Holmes, 1977) and can therefore influence metal levels. Dredging of estuarine and river sediments to facilitate boat traffic is a common practice. Sediment dredging is also an approach in the remediation of polluted sediment (Förstner & Wittmann, 1979). For example, the Kitayama bay, Japan called the "dead sea" in the 1960s owing to the apparent absence of aquatic organisms, has shown a dramatic recovery following dredging (Ueda *et al.*, 1994). The results of a post dredging survey showed a drastic reduction in the levels of heavy metals (Hg and Cd) in bottom sediments and recolonisation of various benthic organisms, although the innermost areas of the bay remain organically polluted (Ueda *et al.*, 1994). Peel

Estuary is dredged annually (Isle of Man Ministry of Transport, pers. comm.).This will undoubtedly affect sediment metal levels. It may not, however, restore the sediment as in the above example because whereas the industrial discharge of metallic contamination into Kitayama Bay has been regulated, spoil heaps and old mine adits continue to deliver contaminated river water into Peel Harbour (Southgate *et al.*, 1983). It will be interesting to see how the suspension and re-deposition of sediment particles during dredging affects the dynamics of adsorption and desorption of metals and hence, metal levels in sediment, interstitial and overlying water in Peel Estuary. No dredging takes place in the Laxey Estuary and the reason for the very high differences in Pb levels recorded between this study and Southgate *et al.*'s remain unclear. Despite the differences, this study confirms the findings of Southgate *et al.* (1983) that elevated metal levels persist in sediments of estuaries associated with former intensive mining regions in the Isle of Man.

3.4.2 Biomonitors

The degree of contamination observed in biomonitors was similar to previously reported values for some of the sites by Southgate *et al.* (1983) for *Mytilus edulis*, and Gibb *et al.* (1996) for *M. edulis* and *Fucus serratus*. Metal levels in *M. edulis* and *F. serratus* indicate a similar trend of contamination as sediment, with Zn and Pb being highly elevated in Peel and Laxey in comparison with Castletown and Ramsey. Some variation, however, occurred in the metal contamination profiles depicted by *M. edulis* and *F. serratus*. The different routes of metal uptake and other factors differentially

affecting these species may elucidate these differences. Being a filter feeder, *M. edulis* takes up heavy metals not only from solution and food, but also from ingestion of inorganic particulate material (Moore, 1971; Rainbow & Phillips, 1993). On the other hand, the underlying assumption in the use of seaweed as indicators of metal contamination is that they essentially respond to the concentration of soluble metal in their surrounding. *M. edulis* was shown to have higher Zn values in Peel than Laxey, while for *F. serratus* Zn values were much higher in Laxey than Peel. It implies that total Zn in water may be higher in Peel but the ratio of dissolved to particulate Zn was higher in Laxey.

The ratio of soluble to particulate metal in an estuary may be influenced by salinity which also affects metal speciation. For example, the more bioavailable forms of Cd and Zn (Cd^{2+} and Zn^{2+} respectively) tend to increase with decreasing salinity (Engel *et al.*, 1981); but, freshwater tends to have low availability of Cu^{2+} due to the chelating properties of its high humic content (Mantoura *et al.*, 1978). Salinity affects the physiology of aquatic organisms leading to alteration in filtration and feeding rates (Phillips, 1980) and may therefore have a direct effect on metal uptake which is variable with metal and organism. Cd uptake by *M. edulis* from solution has been reported to increase with reduced salinity under laboratory conditions (Phillips, 1976a, Jackim *et al.*, 1977). Similarly, *Mytilus galloprovincialis* took up inorganic arsenic faster from solutions of lower salinity (Ünlu & Fowler, 1979). George *et al.* (1978) found that mussels accumulated more Cd in dilute seawater than in 100 % seawater but attributed this increase in Cd uptake to the effect of osmolarity of the surrounding medium. Phillips (1976a) also found a tendency for Cu uptake in *M. edulis* to increase

with a decrease in salinity but this response was very erratic; Pb uptake, however, increased with increased salinity but Zn uptake was not significantly affected. Low salinity was however, found to increase Zn uptake in mussels in experiments where animals were subjected to rapid large-amplitude fluctuations of salinity (Phillips, 1977b).

There were significant within-site variations in levels found in *Mytilus* for Cd in Laxey and for Zn in Peel, as well as an appreciable but non-significant variation of Pb values in the Peel Estuary. In each case metal levels in up-river samples were higher than levels in animals at the mouth. In light of the above laboratory results, it seems that salinity differences may have influenced Cd uptake in Laxey but its effect on metal uptake in Peel is minimal. Hence, the higher up-river values of Zn and Pb in Peel are likely to reflect a gradient of metal abundance and availability from the in-flow river source and possibly sediment. No significant within-site difference in Cu concentration was found at any site, probably confirming previously reported instances of Cu regulation in *M. edulis* (Phillips, 1976a, 1976b; Boyden, 1977). This suggests that the mussel is a poor indicator of Cu but in some instances elevated Cu levels have been found in animals from areas of high Cu contamination (Young *et al.*, 1979, Davies & Pirie, 1980) so they may give a warning of environmental contamination by Cu (Davenport & Redpath, 1984).

Microhabitat variations such as shore level and depth have been shown to affect metal levels in algae and bivalves. Nickless *et al.* (1972) studied the influence of vertical

position of sampling on trace metals in *Fucus vesiculosus* from the Bristol Channel and found a metal-dependent profile. Pb was found to be highest in plants from the upper limit, Cd was highest in the lower limit and Zn was highest in the middle of the vertical range. In the Tamar Estuary, Bryan & Hummerstone (1973a) found that seasonally averaged levels of Zn and Cu in *Fucus vesiculosus* did not change with vertical level on the shore, but Pb was highest at the lowest level. For *Mytilus edulis* and *M. galloprovincialis*, DeWolf (1975) reported that total Hg was generally greater in intertidal animals than subtidal animals. Phillips (1976a) found depth dependent Zn and Cd levels in *M. edulis* from the estuary of the Yarra River, Australia during winter. Stratification of the water column, with low salinity metal rich river water overlying relatively uncontaminated seawater of high salinity accounted for the differences in metal concentrations in the animals at different depths. Both *Fucus serratus* and *Mytilus edulis* in this study were collected from comparable shore heights in each site. The estuaries are almost completely flushed of seawater during low tide so no stratification occurs during winter. Phillips (1980) suggests that the differences in shore level found by DeWolfe (1975) may have resulted from differences in growth rate between intertidal and subtidal animals. Although such differences in growth rate may also occur between intertidal mussels from different locations (Seed, 1976) and needs to be considered in the comparison of metal levels between locations (Davis & Pirie, 1978), it is not thought to have markedly influenced metal levels in mussels in this study. Site-specific microhabitat variations may, however, be important in explaining the metal profiles in *Littorina saxatilis* in this study (see below).

It is known that littorinids are capable of regulating the levels of certain metals especially those essential for metabolic activities such as Cu, Zn, Mn and Fe (Bryan *et al.*, 1983, Webb, 1990). This is confirmed by the very low correlation between levels of Cu in *Enteromorpha* and values in *L. saxatilis* from sites in the Isle of Man. Webb (1990) found similar results for animals in some of the sites used for this work and for samples from sites in Anglesey, Wales. Bryan *et al.* (1983) also obtained evidence of regulation of Cu in *Littorina littorea*. Zn levels may also be regulated but to a lesser extent (Bryan *et al.*,1983) and my results showed a good agreement between Zn levels in *L. saxatilis* and *Enteromorpha*. Pb levels in the snails and *Enteromorpha* showed no significant correlation. Bryan *et al.*, (1983) found that Pb levels in *Fucus serratus* and *Littorina littorea* showed a better relation if the values attributed to particles were subtracted from the total seaweed concentration. They also reported that differences in Pb levels are likely to depend on the extent to which the winkles are allowed to clear their gut contents prior to analysis since the relatively high Pb level in ingested sediment would tend to enhance the concentration of Pb in the winkle. No corrections for possible particulate contribution to metal levels in *Enteromorpha* were made in this study. A considerable particulate contamination might occur in analysis with *Enteromorpha* (despite scrupulous cleaning). Also animals were depurated for only 24 hours before analysis.

Litttorinids are generally considered to be good indicators of contamination with dissolved Cd (Stenner & Nickless, 1974; Manga & Hughes, 1981; Bryan *et al.*, 1983). The high correlation and regression slopes between Cd levels in *L. saxatilis* and *Enteromorpha* show that the same is true for *Littorina saxatilis*.

The contamination levels of Zn, Pb and Cd in *Littorina saxatilis* generally conform to levels in *M. edulis*, *F. serratus* and sediment except for levels in the winkles from Peel which were consistently lower than those from Laxey. Site-specific habitat variation is suggested as the reason for this discrepancy. It appears that the animals from Peel occupy a level of shore where they do not get exposed to as much metals as the above indicators from that site showed. This was confirmed by metal levels in *Enteromorpha intestinalis* collected at the *L. saxatilis* shore level. For this reason, animals from Peel were treated as additional control individuals in subsequent studies and those from Laxey represent the main source of samples for contamination treatments.

CHAPTER FOUR

TOLERANCE TO HEAVY METALS IN *LITTORINA SAXATILIS* FROM A METAL CONTAMINATED ESTUARY IN THE ISLE OF MAN

4.1 INTRODUCTION

The use of aquatic organisms for the monitoring of environmental levels of pollutants has received considerable acceptance over the last 30 years (reviewed by Phillips, 1980; Rainbow & Phillips, 1993; see chapter 3). Acute toxicity tests with aquatic organisms also play a major part in the setting up of regulatory standards for the emission of substances into the environment (Hunt & Anderson, 1993; Widdows, 1993). Standard protocols for selected molluscan species (e.g. *Crassostrea gigas* and *Mytilus edulis*) have been adopted by some regulatory agencies and are currently being implemented in a variety of effluent monitoring and toxicity evaluation programmes (Hunt & Anderson, 1993).

Since factors affecting toxicity include those influencing metal uptake and those affecting the organisms' ability to handle and detoxify accumulated trace metals (Rainbow *et al.*, 1990), tolerance to metals could have implications in environmental management. On the one hand, the setting up of regulatory limits for metals based upon toxicity data extrapolated from test species may be affected by tolerance in a manner similar to the commercial impacts of pesticide resistance (Benjamin & Klaine , 1995). More so, it renders the adoption of a uniform standard for all situations difficult. On the other hand, the mechanisms by which tolerance is achieved (see chapter 6 for details), can result in net metal burdens that may influence the use of organisms for long-term monitoring purposes.

The demonstration of a heightened heritable and specific tolerance to a pollutant in a natural population indicates that the pollutant has exerted a selection pressure on that

population (Luoma, 1977; Hately *et al.*, 1989). The presence of tolerance may be used to identify the relative importance of different potential xenobiotics since a pollutant that was not biologically active, or of a concentration insufficient to produce biological effects, would not exert a selection pressure on an exposed population (Luoma, 1977). Suter (1993) contends that adaptation has generally been ignored in ecological assessment because effects on a population that create enough selective pressure to induce adaptation is assumed to be unacceptable, and because such adaptation is believed to take too long to become ameliorative. The prevalence of widely dispersed propagules or larval stages in many marine organisms also reduces the likelihood of localized selection occurring in many species; it is only likely in species with direct development such as the *Littorina saxatilis* species complex (Janson, 1982; Johannesson *et al.*, 1993, 1995). However, the survival of individual benthic species in severely impacted areas may be the result of the induction of efficient detoxification processes or by the development of metal tolerant strains (Bryan & Langston, 1992). Such is the case with *Nereis diversicolor* in the heavily contaminated Restronguet Creek (Bryan & Hummerstone, 1971,1973b; Grant *et al.*, 1989) and some nematode species in the same estuary (Millward & Grant, 1995).

This chapter examines the tolerance of *Littorina saxatilis* from five sites around the Isle of Man to toxic concentrations of zinc (Zn), copper (Cu), lead (Pb) and cadmium (Cd). The concentration of metals in the tissues of organisms is a function of the processes of uptake, excretion and storage. While elevated concentrations of metals in the tissues may not necessarily be an indication of effects (see Phillips & Rainbow, 1993), tissue metal levels should give a reasonable indication of the presence of metal levels capable of inducing tolerance. On the basis of tissue metal levels in *L. saxatilis*

from different sites in the Isle of Man (details in chapter 3), it is hypothesised that Zn is present in concentrations capable of inducing tolerance in animals from Laxey in comparison with those from Castletown, Derbyhaven, Peel and Ramsey. Furthermore, contamination in the estuary dates back to over two centuries of past mining activity (Southgate *et al.*, 1983, see also Chapter 2). This hypothesis was tested by means of toxicity experiments. Tolerance to Cu, Cd and Pb were similarly assessed to examine whether co-tolerance to these metals exists.

4.2 MATERIALS AND METHODS

Littorina saxatilis were collected from Castletown, Derbyhaven, Laxey, Peel and Ramsey and transported to the laboratory in moist seaweed. Animals of similar size (~10 ± 2 mm shell height) were acclimatised to laboratory conditions (constant temperature room of 10 ±1 ° C, salinity ~34 psu) in tanks containing aerated sea water. They were subsequently transferred to test chambers (Parrish, 1985; Mance, 1987), by suspending samples in labelled nylon mesh bags. Test chambers consisted of 2L conical flasks with rubber stoppers (see Fig 4.1). Each stopper had a hole through which a hollow glass tubing was passed for air delivery. The rubber stoppers also held the lines for mesh identification in place so that the bags were easily retrieved and re-introduced into the flasks during the experiment, and possibly reduced evaporative loss although this was expected to be minimal under the experimental regime in a constant temperature room maintained at 10 °C. Each acclimatization phase lasted 3 days.

After acclimatization to the test chambers, the animals were introduced to test solutions of the appropriate metal concentration. Test concentrations were prepared by diluting a freshly prepared stock solution ($1000mgl^{-1}$) of the respective metal salts in filtered sea water equilibrated to the test temperature. Metal salts used were the sulphates of Zn ($ZnSO_4.7H_2O$), Cu ($CuSO_4.5H_2O$), and Cd ($3CdSO_4.8H_2O$), and Pb nitrate $Pb(NO_3)_2$. The test concentrations were: 5.0, 10.0 and 20.0 mgl^{-1} added Zn and added Pb; 0.5, 1.0 and 2.0 mgl^{-1} added Cu and added Cd. Five replicate treatments of each concentration and controls (no metal added) were used. Replication was achieved by a randomised complete block design, with each block (test flask) containing bagged individuals (ten individuals per bag) from each of the five sites.

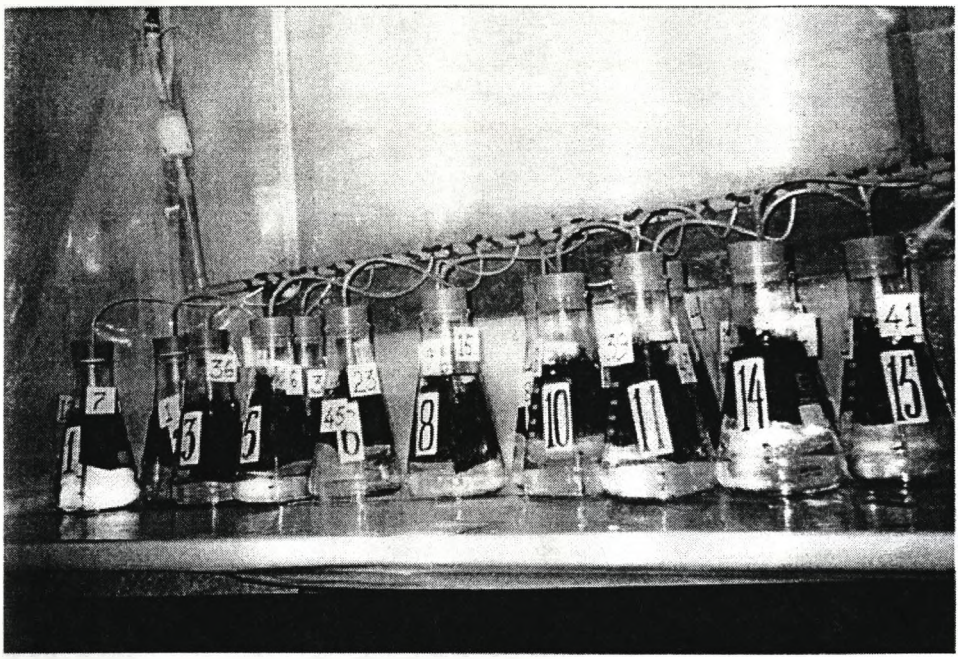

Fig. 4 1 Experimental setup for metal tolerance experiments in place in a C.T. room

The bags were identified by random numbers so that they were examined "blind". The solutions were aerated continuously with air filtered through a glass microfibre filter (Whatman Hepa-cap). All tanks, flasks and bags were acid-washed before use. Test solutions were replaced every other day and individuals were examined for mortality every four days in clean sea water. An individual was considered dead if it failed to withdraw on tactile stimulation. Animals primarily assessed as dead were left in clean seawater for a 24-hr recovery period. In a few cases recovery was observed (within a few hours) and the animals were re-introduced to the appropriate test solution. Those that did not recover were recorded as dead. The test animals were not fed before or during the experiment.

Experiments were run for up to 44 days (except Cu 0.5mgl^{-1} and Cd 2.0 mgl^{-1} = 40 days; Cd 0.5 and 1.0 mgl^{-1} = 28 days) or 100 % mortality.

Data Analysis

Median lethal times (LT_{50}s) for each metal concentration in which over 50 % mortality occurred were obtained by graphical interpolations. Rand & Petrocelli (1985) argued that LC_{50} estimated by graphical interpolation is as accurate as and similar to the LC_{50} obtained by formal statistical methods. Analysis of variance was performed on the LT_{50} values [after $\log_{10}(x+1)$ transformation] to test for differences in the tolerance of animals from various sites. Where significant differences occurred, Tukey HSD multiple comparisons were applied to test the differences between individual means between pairs of sites. Simple correlations were made between LT_{50} values and the tissue burdens of the respective metals.

4.3 RESULTS

Time-mortality curves depicting the response of *Littorina saxatilis* from different sites in the Isle of Man, exposed to various concentrations of Zn, Pb, Cu and Cd are presented in Figs. 4.2, 4.4, 4.6 & 4.8. Of the three Zn concentrations (5, 10 and 20 mg l^{-1} added metal Zn) tested, the difference in response between animals from different sites was most obvious at the "intermediate" (10 mg l^{-1} Zn) concentration (Fig. 4.2b). The curves show that the animals from Laxey were clearly less susceptible to Zn at 10 mg l^{-1} than those from Derbyhaven, Castletown, Peel and Ramsey which showed similar responses. This trend is confirmed by LT_{50} values (Fig 4.3) whereby the mean value for Laxey is double that for other sites. ANOVA showed a significant difference between mean LT_{50}s ($p<0.0001$, see Table 4.1a). Tukey HSD multiple comparisons of mean LT_{50} values (Table 4.1a) show that the animals from Laxey were significantly more tolerant to Zn than animals from all other sites ($p<0.001$). No significant difference was found for animals from Derbyhaven, Castletown, Peel and Ramsey. The tolerance to Zn at 10 mg l^{-1} was highly correlated with the status of metal contamination as reflected in tissue metal burdens ($r=0.985$, $p<0.001$ $n=5$; see Table 4.2).

Mortalities at 5 mg l^{-1} Zn indicate the general pattern of intraspecific differences in tolerance to Zn (Fig. 4.2a). Because mortalities were less than 50% in most replicates (see Appendix B) within the 44-day test period, a full set of LT_{50} estimates could not be obtained for statistical analysis. However, Friedman's test on median time to death (TTD_m) (see Hoare and Davenport, 1994) show a significant difference in median TTD_m between sites (Table 4.3). Tukey-type comparisons detected a significant

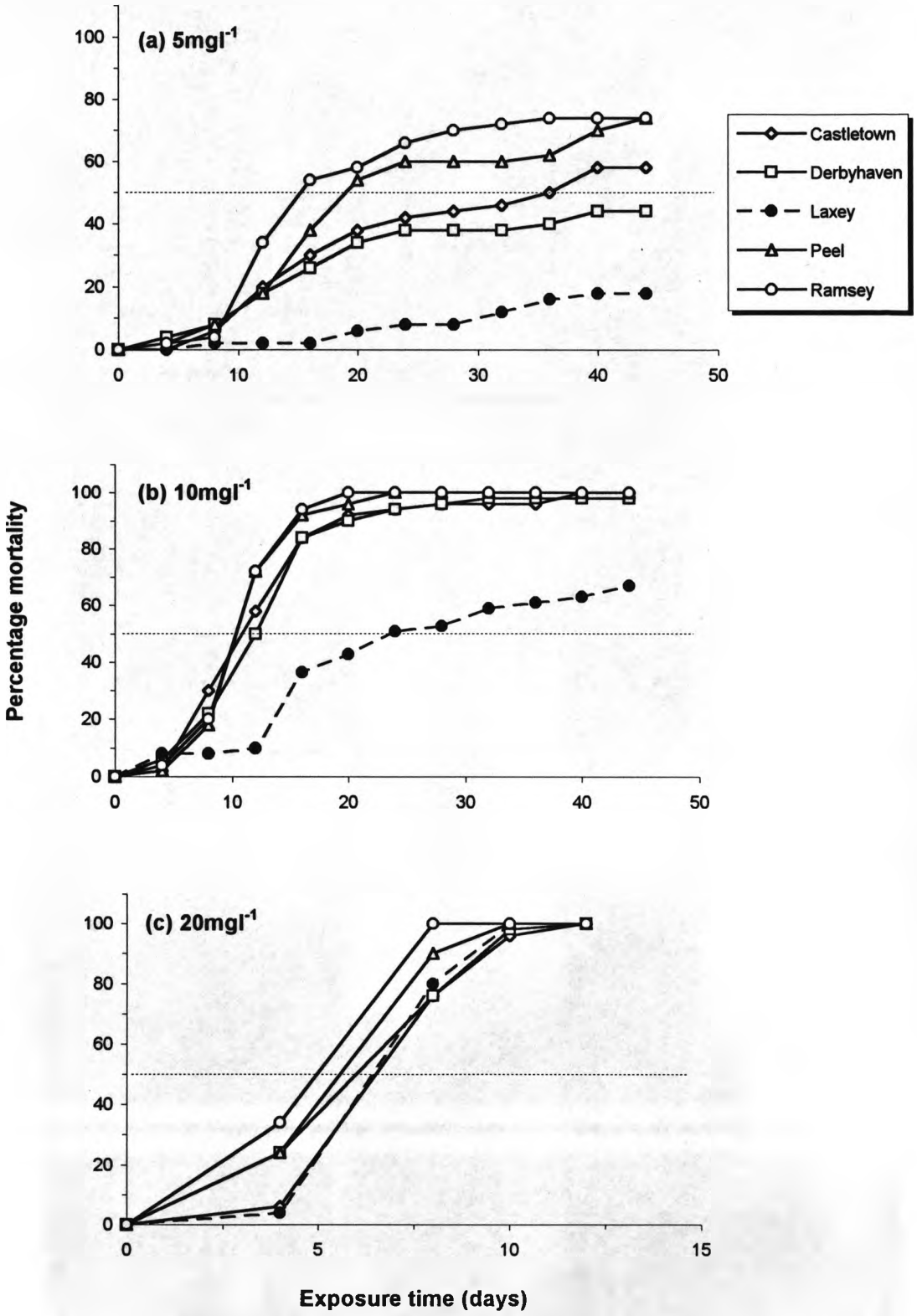

Fig 4.2 Mortality of *Littorina saxatilis* from sites in the Isle of Man exposed to zinc. The plotted values are means of five replicates. a = 5 mgl^{-1}; b = 10 mgl^{-1}; c = 20 mgl^{-1} added zinc (N.B. differences in exposure time). Dotted lines represent 50 percent mortality. Negligible control mortality (max.= 4 %) not plotted.

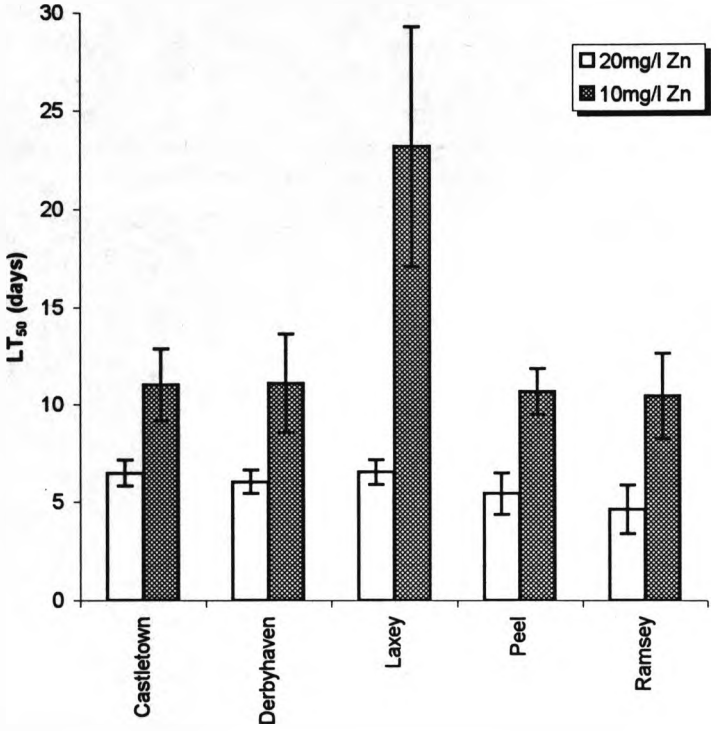

Fig. 4.3 Median lethal times (LT_{50}) of *Littorina saxatilis* from five sites in the Isle of Man exposed to 10mg l^{-1} and 20mg l^{-1} zinc. Values plotted are mean $\pm$ s.d., n = 5.

Table 4.1 Randomised-block Analysis of Variance (ANOVA) on LT_{50} values [log(x+1) transformed] of *Littorina saxatilis* exposed to 10mgl $^{-1}$ and 20 mgl $^{-1}$ zinc. Separate analyses were performed for each metal concentration.

(a) ANOVA for 10 mg l $^{-1}$ Zinc

Source of Variation	df	SS	MS	F	P-value
Site	4	0.3737	0.0934	13.991	<0.0001
Blocks	4	0.0325	0.0081		
Remainder	16	0.1068	0.0067		
Total	24	0.5130			

Significance levels of Tukey HSD comparisons on mean LT_{50}s at 10mgl $^{-1}$ Zn

	Laxey	Castletown	Derbyhaven	Peel
Castletown	***			
Derbyhaven	***	ns		
Peel	***	ns	ns	
Ramsey	***	ns	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001, ns=p>0.05

(b) ANOVA for 20 mg l $^{-1}$ Zinc

Source of Variation	df	SS	MS	F	P-value
Site	4	0.0635	0.0159	4.870	0.009
Blocks	4	0.0273	0.0068		
Remainder	16	0.0522	0.0033		
Total	24	0.1430			

Significance levels of Tukey HSD comparisons on mean LT_{50}s at 20mgl $^{-1}$ Zn

	Laxey	Castletown	Derbyhaven	Peel
Castletown	ns			
Derbyhaven	ns	ns		
Peel	ns	ns	ns	
Ramsey	**	*	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001, ns=p>0.05

Table 4.2 Correlations between tissue levels (μg g^{-1} dry weight) of Zn, Pb, Cu and Cd in *Littorina saxatilis* from various sites and median lethal times on exposure to different concentrations of the respective metals (n=5).

Zinc		Lead		Copper		Cadmium	
Test conc. (mg l^{-1})	r	Test conc. (mg l^{-1})	r	Test conc. (mg l^{-1})	r	Test conc. (mg l^{-1})	r
5	N/A	5	0.424	0.5	-0.290	0.5	N/A
10	0.985***	10	-0.485	1	-0.217	1	-0.251
20	0.405	20	-0.217	2	-0.693	2	0.205

*** $p<0.001$, N/A= insufficient mortalities at metal concentration

Table 4.3 Friedman's test on Median Time to Death (TTD_m) of *Littorina saxatilis* exposed to 5mg l^{-1} zinc.

Site	n	Estimated Median	Sum of Ranks
Castletown	5	42.100	17.0
Derbyhaven	5	44.700	18.0
Laxey	5	48.500	22.5
Peel	5	32.500	11.0
Ramsey	5	30.200	6.5

Grand median = 39.600

S = 14.32 d.f. = 4 p = 0.007** (adjusted for ties)

Significance levels of Tukey-type HSD comparisons on rank sums

	Laxey	Derbyhaven	Castletown	Peel
Derbyhaven	ns			
Castletown	ns	ns		
Peel	ns	ns	ns	
Ramsey	*	ns	ns	ns

*=$p<0.05$; **=$p<0.01$; ***=$p<0.001$, ns=$p>0.05$

difference only between the most tolerant (Laxey) and the least tolerant site (Ramsey) populations ($p<0.05$, Table 4.3).

At 20 mg l^{-1} Zn, the response curves show a broadly similar trend for all sites (Fig. 4.2c) and these are reflected in the mean LT_{50}s (Fig. 4.3). However ANOVA (Table 4.1b) showed a significant difference in mean LT_{50} values between sites. Tukey tests on mean LT_{50} values at 20 mg l^{-1} Zn show that individuals from Ramsey were significantly less tolerant than those from both Laxey ($p<0.01$)and Castletown ($p<0.05$); but, no significant differences occurred between animals from Laxey, Castletown, Derbyhaven and Peel (Table 4.1b). Also, there was no significant correlation between LT_{50} values at 20 mg l^{-1} Zn and the tissue concentrations of Zn ($r=0.045$, $p>0.05$ $n=5$).

Fig. 4.4 shows the inter-site differences in the response of *L. saxatilis* exposed to 5, 10 and 20 mg l^{-1} added Pb. The time-response curves at 5 mg l^{-1} Pb show that animals from Laxey were less susceptible than those from all other sites (Fig. 4.4a). ANOVA on LT_{50} values (Table 4.4a, see Fig. 4.5) showed a significant difference between sites ($p<0.0001$). Tukey HSD comparisons indicate that *L. saxatilis* from Laxey were significantly more tolerant to 5mg l^{-1} Pb than animals from all other sites. Individuals from Derbyhaven and Castletown were also more tolerant than those from Ramsey ($p<0.01$, Table 4.4a). No significant difference was found for comparisons between individuals from Peel:- Derbyhaven, Castletown, or Ramsey. It should be noted that for two replicates in Laxey, 50% mortality was not achieved at the end of the

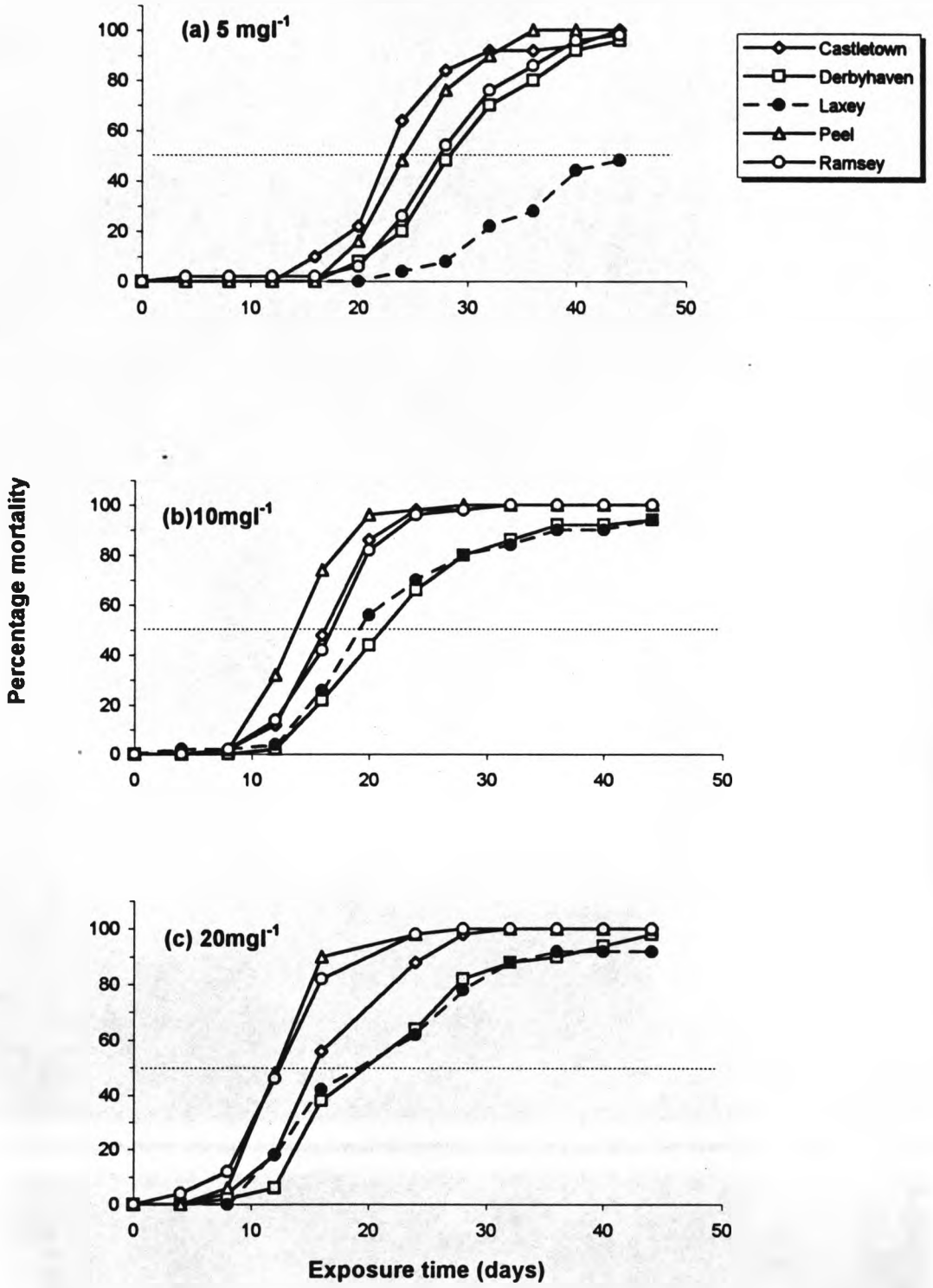

Fig 4.4 Mortality of *Littorina saxatilis* from sites in the Isle of Man exposed to lead. The plotted values are means of five replicates. a = 5 mgl^{-1}; b = 10 mgl^{-1}; c = 20 mgl^{-1} added lead. Dotted lines represent 50 percent mortality. Control mortality for all sites = 0 %, not plotted.

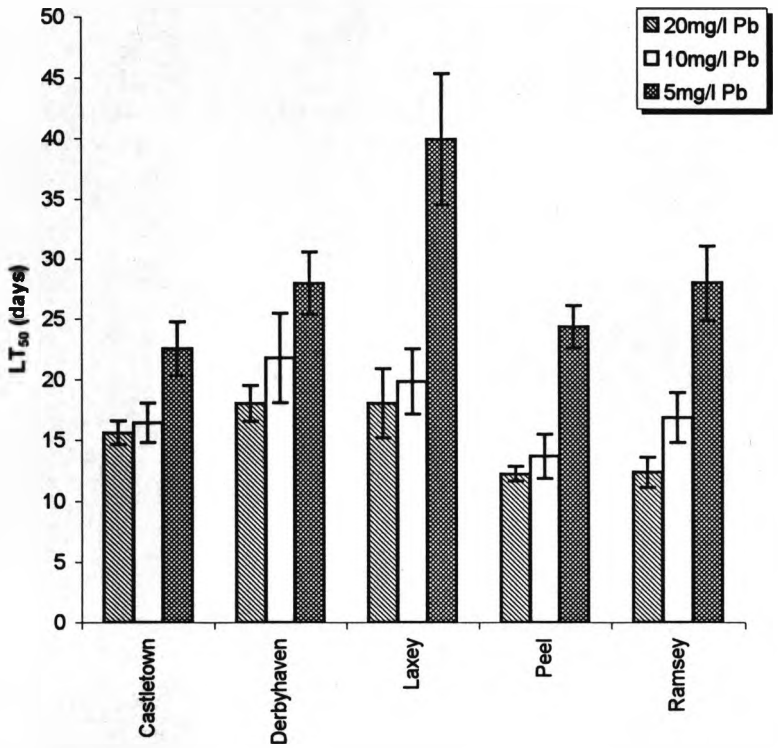

Fig. 4.5 Median lethal times (LT_{50}) of *Littorina saxatilis* from five sites in the Isle of Man exposed to 5mg l^{-1} , 10 mg l^{-1} and 20 mg l^{-1} lead. Values plotted are mean $\pm$ s.d., n = 5.

Table 4.4 Randomised-block Analysis of variance (ANOVA) on LT_{50} values [log(x+1) transformed] of *Littorina saxatilis* exposed to 5mgl^{-1}, 10mgl^{-1} and 20mgl^{-1} lead. Separate analyses were performed for each metal concentration.

(a) ANOVA for 5mg l^{-1} lead

Source of Variation	df	SS	MS	F	P-value
Site	4	0.1674	0.0419	45.504	<0.0001
Blocks	4	0.0251	0.0063		
Remainder	16	0.0147	0.0009		
Total	24	0.2072			

Significance levels of Tukey HSD comparisons on mean LT_{50}s at 5mgl^{-1} Pb

	Laxey	Derbyhaven	Ramsey	Peel
Derbyhaven	***			
Ramsey	***	ns		
Peel	***	ns	ns	
Castletown	***	**	**	ns

*=p<0.05; **=p<0.01; ***=p<0.001, ns=p>0.05

(b) ANOVA for 10mg l^{-1} lead

Source of Variation	df	SS	MS	F	P-value
Site	4	0.1077	0.0269	8.040	0.001
Blocks	4	0.0041	0.0010		
Remainder	16	0.0536	0.0034		
Total	24	0.1654			

Significance levels of Tukey HSD comparisons on mean LT_{50}s at 10mgl^{-1} Pb

	Derbyhaven	Laxey	Ramsey	Castletown
Laxey	ns			
Ramsey	ns	ns		
Castletown	ns	ns	ns	
Peel	***	**	ns	ns

*=p<0.05; **=p<0.01; ***=p<0.001, ns=p>0.05

(c) ANOVA for 20mg l^{-1} lead

Source of Variation	df	SS	MS	F	P-value
Site	4	0.1213	0.0303	30.129	<0.0001
Blocks	4	0.0150	0.0037		
Remainder	16	0.0161	0.0010		
Total	24	0.1524			

Significance levels of Tukey HSD comparisons on mean LT_{50}s at 20mgl^{-1} Pb

	Derbyhaven	Laxey	Castletown	Ramsey
Laxey	ns			
Castletown	ns	ns		
Ramsey	***	***	**	
Peel	***	***	**	ns

*=p<0.05; **=p<0.01; ***=p<0.001, ns=p>0.05

experiment (see appendix B) and the LT_{50} values were conservatively estimated by assuming that all animals would die at the next examination time.

The tolerance curves for 10- and 20 mg l^{-1} Pb were generally similar probably because of the saturation of the solutions above 5mg l^{-1} Pb in seawater (see Mance, 1987). At 10 mg l^{-1} Pb, animals from Derbyhaven and Laxey showed similar levels of mortality which were lower than those from Ramsey, Castletown and Peel (Fig. 4.4b). LT_{50} values for 10mg l^{-1} Pb are presented in Fig. 4.5 and there was a significant difference between sites (ANOVA, $p<0.001$, Tale 4.4b). Tukey tests only showed significantly higher LT_{50} values for Derbyhaven and Laxey individuals in comparison with those from Peel ($p<0.01$, Table 4.4b). Figs 4.4c and 4.5 indicate that at 20 mg l^{-1} Pb animals from Derbyhaven and Laxey showed the highest tolerance (Fig. 4.2c). ANOVA showed significant differences between sites in mean LT_{50} values at 20 mg l^{-1} ($p<0.0001$, Table 4.4c). Tukey HSD multiple comparisons on mean LT_{50} values at 20mg l^{-1} gave the following inference:

Derbyhaven =Laxey =Castletown >Ramsey =Peel

Correlations between LT_{50} values for exposure to Pb and tissue Pb burdens, none of which were significant, are given in Table 4.2.

In all test concentrations of Cu (0.5, 1 and 2 mg l^{-1}) animals from Laxey and Ramsey were the most and least tolerant respectively, but individuals from other sites did not show any consistent pattern (Fig. 4.6). Mean LT_{50} values for tolerance to Cu are presented in Fig 4.7 and reduced values were obtained with increasing concentration of Cu for all sites. Analysis of variance showed significant differences between sites for all

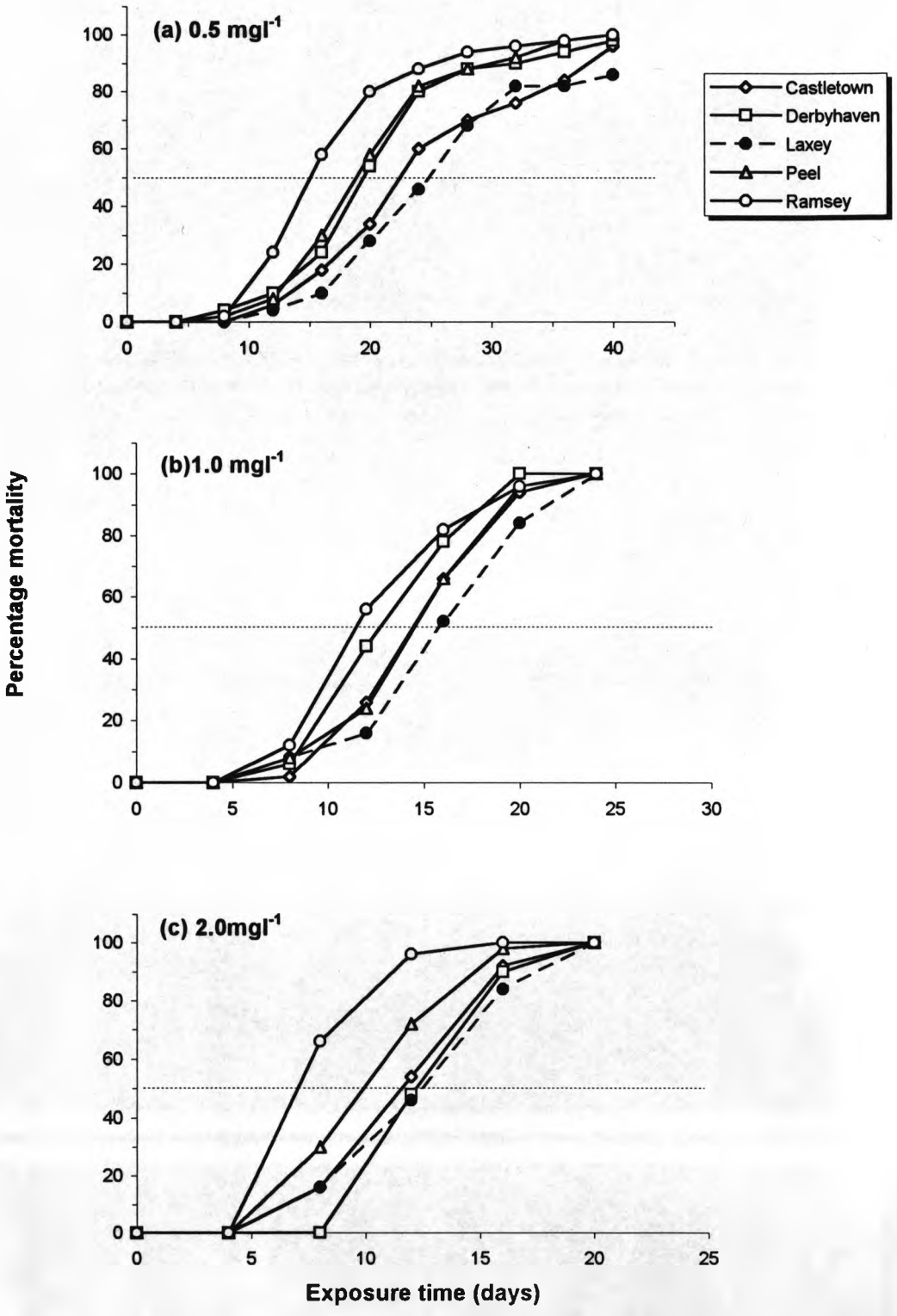

Fig 4.6 Mortality of *Littorina saxatilis* from sites in the Isle of Man exposed to copper. The plotted values are means of five replicates. a = 0.5 mgl^{-1}; b = 1 mgl^{-1}; c= 2 mgl^{-1} added copper (N.B. differences in exposure time). Dotted lines represent 50 percent mortality. Control mortality (0 %) not plotted.

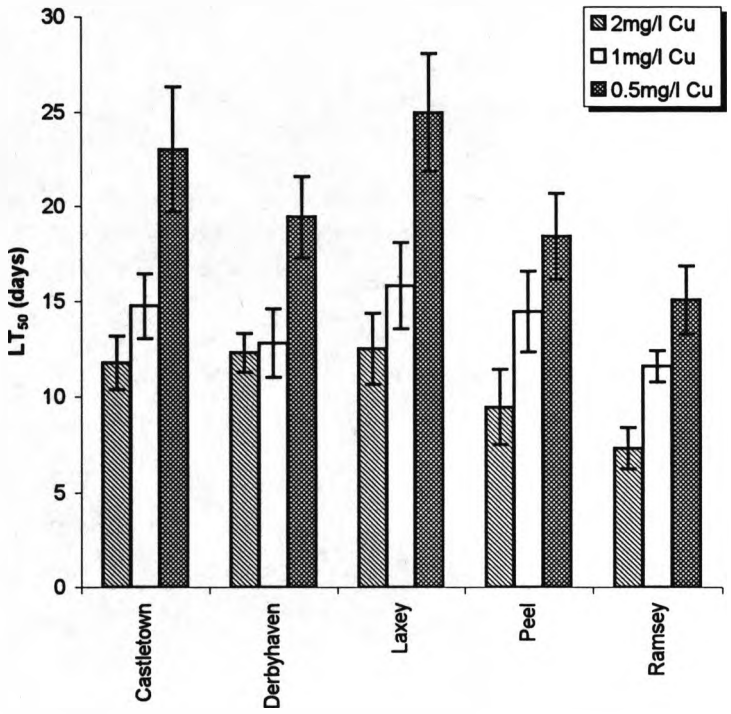

Fig. 4.7 Median lethal times (LT_{50}) of *Littorina saxatilis* from five sites in the Isle of Man exposed to 0.5mg l^{-1}, 1mg l^{-1} and 2mg l^{-1} copper. Values plotted are mean ± s.d., n = 5.

Table 4.5 Randomised-block Analysis of Variance (ANOVA) on LT_{50} values [log(x+1) transformed] of *Littorina saxatilis* exposed to 0.5mgl^{-1}, 1mgl^{-1} and 2 mgl^{-1} copper. Separate analyses were performed for each metal concentration.

(a) ANOVA for 0.5mg l^{-1} Copper

Source of Variation	df	SS	MS	F	P-value
Site	4	0.1336	0.0334	12.531	<0.0001
Blocks	4	0.0116	0.0029		
Remainder	16	0.0427	0.0027		
Total	24	0.1879			

Significance levels of Tukey HSD comparisons on mean LT_{50}s at 0.5mgl^{-1} Cu

	Laxey	Castletown	Derbyhaven	Peel
Castletown	ns			
Derbyhaven	*	ns		
Peel	**	ns	ns	
Ramsey	***	***	*	ns

*=p<0.05; **p<0.01; ***=p<0.001, ns=p>0.05

(b) ANOVA for 1mg l^{-1} Copper

Source of Variation	df	SS	MS	F	P-value
Site	4	0.0490	0.0122	8.416	<0.001
Blocks	4	0.0293	0.0073		
Remainder	16	0.0233	0.0015		
Total	24	0.1016			

Significance levels of Tukey HSD comparisons on mean LT_{50}s at 1mgl^{-1} Cu

	Laxey	Castetown	Peel	Derbyhaven
Castetown	ns			
Peel	ns	ns		
Derbyhaven	*	ns	ns	
Ramsey	***	**	*	ns

*=p<0.05; **p<0.01; ***=p<0.001, ns=p>0.05

(c) ANOVA for 2mg l^{-1} Copper

Source of Variation	df	SS	MS	F	P-value
Site	4	0.1657	0.0414	12.310	<0.0001
Blocks	4	0.0135	0.0034		
Remainder	16	0.0539	0.0034		
Total	24	0.2331			

Significance levels of Tukey HSD comparisons on mean LT_{50}s at 2mgl^{-1} Cu

	Laxey	Derbyhaven	Castletown	Peel
Derbyhaven	ns			
Castletown	ns	ns		
Peel	*	ns	ns	
Ramsey	***	***	***	ns

*=p<0.05; **p<0.01; ***=p<0.001, ns=p>0.05

concentrations: 0.5 mg l^{-1} Cu ($p<0.0001$), 1mg l^{-1} Cu ($p<0.01$), 2mg l^{-1} Cu ($p<0.0001$) (Table 4.5). Individuals from Laxey were found to possess higher LT_{50}values at 0.5mgl^{-1} Cu than animals from all other sites except Castletown (Table 4.5a). Only two populations had significantly lower tolerance at 1mg l^{-1} Cu (Derbyhaven, $p<0.025$; Ramsey, $p<0.001$) and 2mg l^{-1} Cu (Peel, $p<0.05$; Ramsey, $p<0.001$) in comparison with animals from Laxey (Table 4.5b & c). Also significantly lower mean LT_{50}s at 1 mg l^{-1} Cu were found for animals from Ramsey in comparison with samples from Castletown ($p<0.01$) and Peel ($p<0.05$). Animals from Derbyhaven ($p<0.001$) and Castletown ($p<0.001$) also had significantly higher LT_{50} values at 2mgl^{-1} Cu than those from Ramsey. All correlations between LT_{50} values for exposure to Cu (0.5, 1 and 2mg l^{-1}) and tissue Cu concentrations were negative and none were significant (see Table 4.2).

Tolerance curves for the Cd toxicity experiments are presented in Fig 4.8. Mortalities at 0.5 mg l^{-1} Cd were low (Fig 4.8a), only a few replicates showed up to 50% mortality over the study period (appendix B). Friedman's test showed no significant differences on median TTD_m between sites for tolerance to 0.5mg l^{-1} Cd (Table 4.6). Tolerance curves for 1 mg l^{-1} Cd indicate that animals from Derbyhaven were the least susceptible (Fig 4.8b) and they also had the lowest mean LT_{50} values (Fig 4.9). However, ANOVA on mean LT_{50}s for 1mg l^{-1} Cd did not produce a significant difference between sites (Table 4.7a). Susceptibility to Cd at 2 mg l^{-1} was highest for animals from Peel and lowest for those from Laxey (see Figs. 4.8c, & 4.9). ANOVA showed a significant difference between sites of LT_{50} values at this concentration (Table 4.7b). Tukey HSD comparisons on mean LT_{50}s found that individuals from Laxey, Ramsey

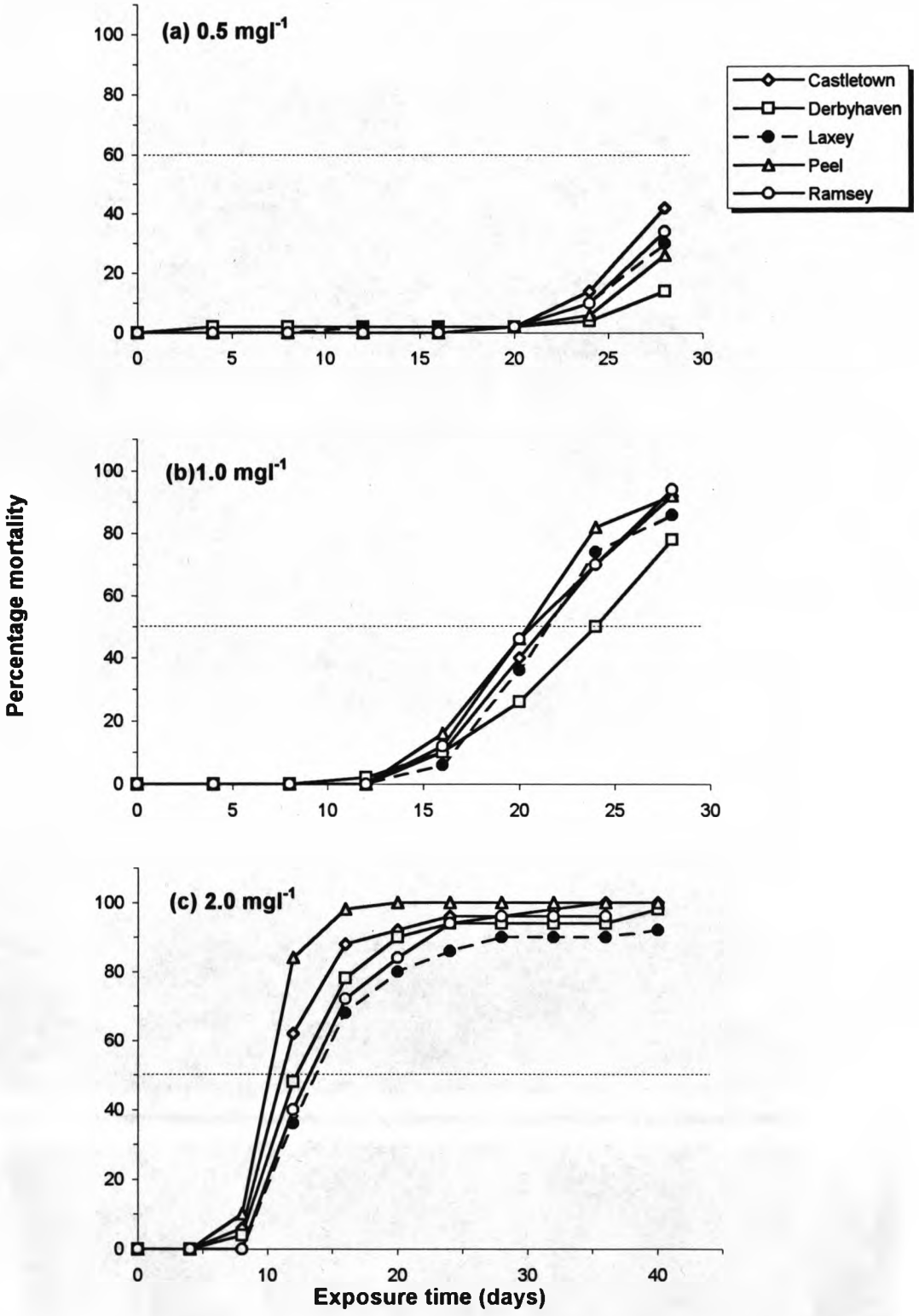

Fig 4.8 Mortality of *Littorina saxatilis* from sites in the Isle of Man exposed to cadmium. The plotted values are means of five replicates. a = 0.5 mgl^{-1}; b = 1 mgl^{-1}; c= 2 mgl^{-1} added cadmium (N.B. differences in exposure time).Dotted lines represent 50 percent mortality. Control mortality (0 %) not plotted.

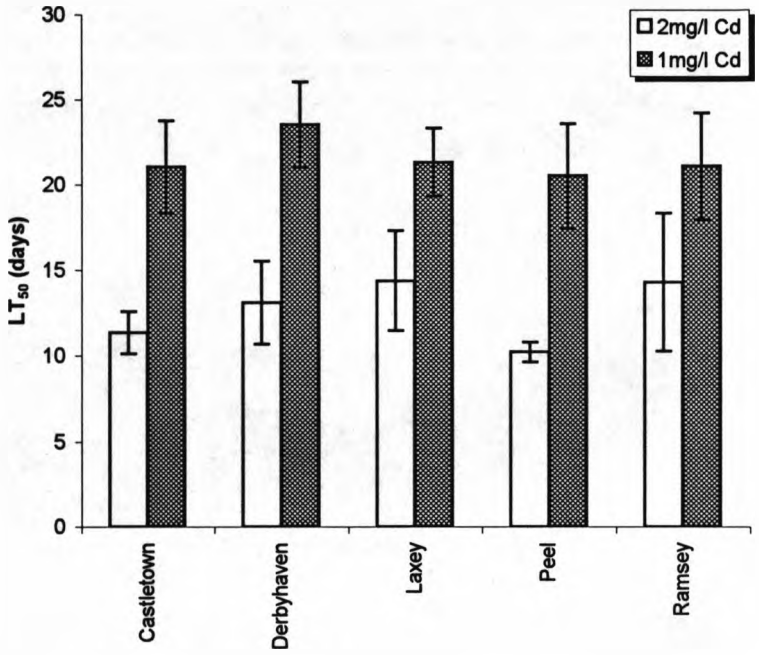

Fig. 4.9 Median lethal times (LT_{50}) of *Littorina saxatilis* from five sites in the Isle of Man exposed to 1mg l^{-1} and 2mg l^{-1} cadmium. Values plotted are mean $\pm$ s.d., n = 5.

Table 4.6 Friedman's test on Median Time to Death (TTD_m) of *Littorina saxatilis* exposed to 0.5mg l^{-1} cadmium.

Site	n	Estimated Median	Sum of Ranks
Castletown	5	29.000	12.0
Derbyhaven	5	29.000	17.5
Laxey	5	29.000	16.0
Peel	5	29.000	14.5
Ramsey	5	29.000	15.0

Grand median = 29.000

S = 3.47 d.f. = 4 p = 0.482 (adjusted for ties)

Table 4.7 Randomised-block Analysis of variance (ANOVA) on LT_{50} values[log(x+1) transformed] of *Littorina saxatilis* exposed to 1mg l^{-1} and 2mg l^{-1} cadmium. Separate analyses were performed for each metal concentration.

(a) ANOVA for 1mg l^{-1} cadmium

Source of Variation	df	SS	MS	F	P-value	$F_{0.05}$
Site	4	0.0102	0.0026	2.917	0.06	3.007
Blocks	4	0.0392	0.0098			
Remainder	16	0.0140	0.0009			
Total	24	0.0634				

(b) ANOVA for 2mg l^{-1} cadmium

Source of Variation	df	SS	MS	F	P-value
Site	4	0.0638	0.0159	7.399	0.001
Blocks	4	0.0706	0.0176		
Remainder	16	0.0345	0.0022		
Total	24	0.1688			

Significance levels of Tukey HSD comparisons on mean LT_{50}s at 2mg l^{-1} Cd

	Laxey	Ramsey	Derbyhaven	Castletown
Ramsey	ns			
Derbyhaven	ns	ns		
Castletown	*	ns	ns	
Peel	**	**	**	ns

*=p<0.05; **p<0.01; ***=p<0.001, ns=p>0.05

and Derbyhaven were more tolerant to 2mg l^{-1} Cd than those from Peel ($p<0.01$). No significant difference was found between animals from Castletown and Peel.

Correlations between tissue metal concentrations and LT_{50}s were negative for 1mg l^{-1} Cd ($n=5$, $r= -0.251$ $p>0.05$) and positive for 2mg l^{-1} Cd ($n=5$, $r=0.205$, $p>0.05$) but neither were significant (see Table 4.2).

4.4 DISCUSSION

Prior exposure to Zn has been found to result in enhanced tolerance to lethal concentrations of Zn in *Littorina saxatilis*. Animals from Laxey which had the highest Zn levels were significantly more tolerant on exposure to 10 mg l^{-1} Zn than animals from Castletown, Derbyhaven, Ramsey and Peel. Robinson (1985), Webb (1990) and Jones (1995) have similarly reported Zn tolerance in *L. saxatilis* from Dulas Bay which is contaminated by acid-mine drainage in comparison with "uncontaminated" control sites in Anglesey, Wales. Webb (1990) also tested animals from some of the sites in the Isle of Man used in this study and found enhanced Zn tolerance in animals from Laxey. However, he found that enhanced tolerance occurred at a lower concentration (2.5mg l^{-1} Zn) but that at higher concentrations (5mg l^{-1} and 10mg l^{-1} Zn) a "shift" occurred such that those from faunistically rich "clean" sites that were apparently more healthy, exhibited more tolerance than those from "contaminated" sites. This phenomenon was observed for populations from Anglesey in response to Cu tolerance and both Anglesey and Isle of Man populations in response to Zn.

Tolerance to Zn by *L. saxatilis* in this study was demonstrated at 10mg l^{-1} but not at 20mg l^{-1}. However, rather than a "shift" in tolerance, the responses to 20mg l^{-1} Zn by animals from most sites were similar, indicating that the mechanisms by which tolerance was achieved at the lower concentration were unable to cope with the excess metal. On the basis of results of laboratory experiments Roesijadi & Fellingham (1987) constructed a preliminary conceptual model which relates changes in metal tolerance to the magnitude of metal exposure. The model qualitatively separated the response of individual organisms to metal exposure into four states: (I) responses to natural

background conditions and low but elevated, exposures: no discernible difference between control and exposed organisms; (II) exposure results in bioaccumulation; protective systems are mobilised; protection against toxicity conferred; (III) maximal participation of protective systems; upper limit of compensatory response; (IV) severe exposure: acutely toxic, mobilisation of detoxification systems not sufficient to protect essential metabolic pathways. The intraspecific responses to Zn obtained for *Littorina* in this study broadly conform to this model. The elevated Zn level in Laxey means that the animals from that estuary have protective systems mobilised at the time of sampling (stage II). The concentration at which tolerance was demonstrated (10 mg l^{-1} Zn) conformed to stage III, but at the higher test concentration, stage IV is reached whereby the protective systems are overwhelmed by the severity of exposure.

Webb (1990) found that the Anglesey populations of *L. saxatilis* generally had higher tolerance to Zn than the populations from the Isle of Man. Jones (1995) examined tolerance to Zn in the Anglesey populations at the same time at which my experiments were conducted (for 5mg l^{-1} and 10 mg l^{-1} Zn); the only difference being that the animals from Anglesey were collected a day earlier than those from the Isle of Man sites. Both experiments were conducted at the Port Erin Marine Laboratory, Isle of Man, making the results directly comparable (Webb's experiments were conducted in Manchester). No marked differences were found, but the Isle of Man populations were relatively more tolerant to Zn than the populations from Anglesey. It was expected that animals from Laxey would show less susceptibility than those from Dulas Bay (the contaminated site in Anglesey) because individuals in Laxey are exposed to higher levels of Zn. The interesting point, however, is that the Dulas Bay animals also showed more tolerance to Zn than those from other sites on Anglesey and Isle of Man

control sites. It is possible that the differences in transit time which was longer for the Isle of Man samples in Webb's case, but the reverse in the case of Jones (1995) and my work could have influenced the results. That the transit time had a qualitatively inverse relationship with the relative susceptibilities of individuals between the two experimental series raises the question of the effects of stress during transportation on the overall results of tolerance experiments. The acclimatization period is expected to minimise any such effects, but it seems likely that a longer acclimatization period would be required if test animals are collected from considerably distant locations. Jones (1995) suggests that the use of different metal salts (Zn chloride and Zn sulphate) for the two experiments, and some differences in the experimental protocol such as frequency of renewal of test solutions, may account for some of the differences obtained. While that may be relevant in the actual experimental concentration at which tolerance was found which were lower in Webb's work, differences in time (possibly seasons) is likely to explain more of the variability in the findings. Webb's experiments for the Isle of Man populations and the Anglesey populations were conducted at different times. Roesijadi & Fellingham (1987) observed differences in the response of mussels from the same site (Sequim Bay, USA) exposed to mercury at different times. The differences were explained on the basis of seasonal differences in condition of the two groups of mussels; the first group having been collected and utilised in late spring, while those in the second group were collected and utilised in late winter.

Several workers have demonstrated tolerance to heavy metals resulting from in-situ exposure of organisms to sub-lethal contamination. Bryan & Gibbs (1983) reported the presence of enhanced tolerance in a variety of species from Restronguet Creek in

comparison with nearby "uncontaminated" estuaries: *Nereis diversicolor* and *Carcinus meanas* to Zn and Cu; *Nephthys hombergi*, *Corophium volutator* and *Scrobicularia plana* to Cu. The nematode *Tripyloides marinus* from Restronguet Creek also has elevated tolerance to Cu and Zn (Millward & Grant, 1995). Abdullah & Ireland (1986) studied the response of *Nucella lapillus* populations from the Bristol Channel and Cardigan Bay to Cd toxicity. They found that populations with higher initial Cd burdens exhibited the least susceptibility to acute Cd exposures.

The results of this study show that *L. saxatilis* from Laxey were also more tolerant to Pb at 5mg l^{-1} Pb than animals from all other sites. However, while tolerance to 10mgl^{-1} Zn was significantly positively correlated with tissue burdens of Zn, Pb tolerance at 5mg l^{-1} Pb was not significantly correlated with tissue Pb levels. This suggests that there may be co-tolerance between Zn and Pb in this species, possibly the presence of Zn tolerance predisposing the animals to Pb tolerance. However, since the highest Pb levels were also obtained in the animals from Laxey, the possibility of tolerance to Pb being independent of Zn tolerance cannot be ruled out. The mechanistic basis of Pb tolerance in *L. saxatilis* is examined further in metal uptake studies (chapter 6).

Tolerance to more than one metal has been reported in other species; in some the tolerance between metals is linked but in others it is independent. *N. diversicolor* from Restronguet Creek where Zn and Cu are present at levels likely to have biological effects (Bryan & Gibbs, 1983, Hately *et al.*, 1989) have developed Zn and Cu tolerance by separate means (see Bryan & Hummerstone, 1971,1973b). The distribution of populations of *N. diversicolor* exhibiting tolerance to Zn and Cu supports the findings that they are not linked (Grant *et al.*, 1989). Pb tolerance in *N.*

diversicolor also appears to have developed separately but silver tolerance was dependent upon the presence of Cu (and probably Pb) tolerance (Bryan, 1976b). Brown (1978) reported co-tolerance to Pb by a Cu-tolerant population of *Asellus meridianus* from the River Hayle. A Pb-tolerant population of the same species from the Gannel did not, however, possess co-tolerance to Cu. Roesijadi & Fellingham (1987) found that pre-exposure of *Mytilus edulis* in the laboratory to Cu, Cd and Zn conferred enhanced tolerance to Hg. However, enhanced tolerance to Cd in the fiddler crab, *Uca pugilator* was metal-specific and did not confer increased resistance to mercury (Weis & Kim, 1986). Tolerance to Zn in *L. saxatilis* did not appear to have conferred tolerance to Cu or Cd. Webb (1990) found tolerance to both Zn and Cu in animals from Dulas Bay in comparison with those from Aberffraw and Traeth Bychan. However, elevated levels of both metals occur in Dulas Bay and the enhanced resistance was suggested to have developed independently.

This discussion has so far centred on the assumption that metal tolerance results only as a result of prior exposure to sublethal levels but this may not always be the case. There are other factors which on their own or superimposed on prior exposure could result in intra-specific differences in metal tolerance. Selection for increased metal tolerance might occur (reviewed by Nevo *et al.*, 1983). However, even when tolerance is a mere expression of phenotypic plasticity, ecological differences at different sites and other factors capable of causing variation in the condition of animals from different locations can cause differences in response to metals. Differences in temperature and salinity regimes (Chapman *et al.*, 1985; Kitching *et al.*, 1987) and the incidence of parasitism (Sullivan *et al.*, 1977; Guth *et al.*, 1984) are likely to affect the condition and susceptibility to metal stress in gastropods. Although at 10

mg l^{-1} Zn, the only significant elevation in tolerance was exhibited by animals from Laxey; at 5mg l^{-1} Pb, in addition to animals from Laxey which showed significant tolerance than those from all other sites, animals from Castletown and Derbyhaven also showed significantly lower susceptibility than those from Ramsey. The resistance in the latter populations is neither related to tissue Pb levels nor an expression of co-tolerance to Zn. Furthermore, even at those metal concentrations (e.g. 20 mg l^{-1} Zn, 10mg l^{-1} Pb, 0.5mg l^{-1} Cu and 2 mg l^{-1} Cd) where no population showed a significant across-the-board tolerance, some significant differences were obtained (see e.g. Tables 4.1b, 4.5a, 4.7b) which may have resulted from intrinsic factors unrelated to prior metal exposure. Tolerance to generalised stressors is examined in Chapter 5.

CHAPTER FIVE

DIFFERENCES IN TOLERANCE TO EXTREME SALINITY AND DESICCATION STRESS IN *LITTORINA SAXATILIS* FROM A METAL-CONTAMINATED AND UNCONTAMINATED SITES

5.1 INTRODUCTION

Pollution-induced stress indices at various levels of organisation, from sub-cellular to organismal level, have been investigated as a means of identifying and monitoring environmental contamination (e.g. Bayne *et al.*, 1979; Sheeham *et al.*, 1984; Graney & Giesy, 1986; 1988; see Giesy *et al.*, 1988; Widdows & Donkin, 1992 for reviews). Moving up through the hierarchy of biological organization, relevance and realism increase, but repeatability and standardization decrease (Maltby & Naylor, 1990). Biochemical measures of toxic effects include RNA/DNA ratio (Kearns & Atchinson, 1979; Wilder & Stanley, 1983; McKee & Knowles, 1986), protein content (McKee & Knowles, 1986), lysosomal membrane stability (e.g. Moore & Stebbing, 1976; Moore *et al.*, 1982; Versteeg & Giesy, 1985), concentration of specific enzymes (e.g. Jackim *et al.*, 1970; Versteeg & Giesy, 1985) and adenylate energy charge (e.g. Giesy *et al.*, 1983; Heath, 1984; Sklar & McKee, 1984). Since changes elicited by toxic materials must occur at the biochemical, cellular and tissue levels of organisation before effects will be observed at the organismal level, such measures have been suggested as potential short-term, functional measures of effects which can be used to predict the effects of chronic exposures at higher levels of organisation (McKee & Knowles, 1986; Giesy & Graney, 1989). They also have the advantage of a relatively short response time (Giesy *et al.*, 1988; Giesy & Graney, 1989, Maltby & Naylor, 1990). However, interpreting the ecological relevance of tests based on physiological, cellular and subcellular responses poses serious problems (Maltby & Naylor, 1990).

An organismal level response, the "scope for growth" (SFG) response, is thought to combine both quick response and ecological relevance (Bayne *et al.*, 1979). Widdows

et al. (1995) states that "to date, many field studies including international workshops organised by the Group of Experts on the Effects of Pollutants (GEEP) of the Intergovernmental Oceanographic Commission (IOC) have consistently shown that the 'scope for growth' (SFG) is one of the most sensitive measures of pollution induced stress". The SFG assay evaluates the effects of stress at the level of individual energy budgets and reflects the balance between energy acquisition (feeding and digestion) and energy expenditure (metabolism and excretion), and thus provides an instantaneous measure of the energy status of an animal (Naylor *et al.*, 1989; Maltby & Naylor, 1990; Widdows *et al.*, 1995; see Widdows & Donkin, 1992; Widdows, 1993 for reviews).

Viarengo *et al.* (1995) have recently proposed the use of stress on stress (SOS) response (in mussels) as an index of general stress at the organismal level which can be applied as a monitoring tool for the assessment of contaminated coastal areas. The SOS response is predicated on the definition of stress by Bayne (1986) as "a measurable alteration of biochemical and physiological parameters induced by an environmental change which results in a reduced capacity of the individual to adapt to further environmental change". Viarengo *et al.* (1995) observed that short-term exposure (3 days) of mussels to sublethal concentrations of heavy metals and organic xenobiotics resulted in reduced survival time in air; and that there was a good agreement between the survival data and results of lysosomal membrane stability examination. Tolerance to heavy metals may lead to increased susceptibility to other stressors as a physiological trade-off. The stress on stress response could be extended to evaluate this.

In another context, the operation of a general "fitness factor" could render a population relatively less susceptible to a wide range of stressors including heavy metals. Fisher (1977) noted that estuarine diatoms possess a heightened resistance to a wide range of pollutants and suggested that this resistance was not due to tolerance induced by previous exposure, but due to a more general ability to withstand environmental perturbations. Fisher's comparison of oceanic and estuarine diatoms represent two extremes. However, Webb (1990) obtained evidence that *L. saxatilis* from relatively uncontaminated but faunistically rich sites had relatively higher tolerances to heavy metals (at high exposure concentrations), as well as salinity and desiccation stress, than those from sites contaminated with metals. Since factors other than previous exposure to metals may confer tolerance to metals, tolerance to general stressors needs to be assessed to put metal tolerance into perspective. Tolerance to natural stressors (salinity and desiccation) was examined in *L. saxatilis* from the sites used in the metal tolerance experiments in an attempt to answer the questions:

1. Has tolerance to Zn (and Pb) resulted in a physiological trade-off ?
2. Is the observed metal tolerance an expression of a general stress tolerance ?

5.2 MATERIALS AND METHODS

The protocol of collection/acclimatization of test animals and experimental design for the salinity tolerance experiments were similar to that used for assessing acute metal tolerance (see 4.2, Fig, 4.1). The experiments were run in November/December 1994 and *Littorina saxatilis* were collected from five sites (Castletown, Derbyhaven, Laxey, Peel and Ramsey). Individuals of a standard size (~10 $\pm$ 2 mm shell height) were acclimatized to test conditions (10 $\pm$ 1 ° C) as described in section 4.2. In place of metal solutions, the treatments were hypo-/hyper-saline water. Salinities tested were 0, 17 and 60 practical salinity unit (psu); and 100 % sea water (34 psu) was used as control. 0 psu was obtained from double distilled water, 17 psu was prepared as a 1:1 dilution of sea water in double distilled water and 60 psu was prepared by the addition of a commercial aquarium salt (Instant Ocean, Aquarium Systems, France) in sea water. Aeration was maintained throughout the experiment with filtered air and the animals were not fed before or during the experiment. Mortality was examined every four days. At 0 and 60 psu the experiments were terminated on total mortality, obtained on the 12th day but the 17 psu treatment and controls were followed for a further 12 days.

Tolerance to desiccation was tested by aerial exposure of five replicates of 10 bagged individuals (per replicate) at 24 °C (variations of up to 3 °C were recorded). Replicates were placed in a completely random order in a controlled-temperature room and the experiment was run for 44 days. Mortalities were examined every four days by placing

animals in sea water for 30 minutes. Live animals usually emerged from the shells or showed opercular response to gentle mechanical stimulation.

Data Analysis

Median lethal times (LT_{50}s) for each salinity exposure and for the desiccation experiment were obtained by graphical interpolations. Analysis of variance was performed on the LT_{50} values [after $\log_{10}(x+1)$ transformation] to test for differences in tolerance of animals from various sites (see 4.2). Where significant differences occurred, Tukey HSD multiple comparisons were applied to test the differences between individual means between pairs of sites. Relationships between tolerance to salinity/desiccation and metal tolerance were assessed by simple correlations between the LT_{50} values.

5.3 RESULTS

No control mortalities were observed throughout the study period and the mortalities at 50 % seawater (17 psu) were also very low with the highest average mortality of only 14 % at the termination of the experiments after 24 days exposure period (Fig 5.1a). Time-mortality curves for exposures to 0 psu and 60 psu are presented in Fig. 5.1b and 5.1c. In both extreme salinity treatments, no mortalities were observed on the 4th day but huge mortalities were recorded on day-8, and by day-12 there was total mortality in most cases. The order of tolerance was also similar in both 0 psu and 60 psu with an indication that *Littorina saxatilis* from Laxey were the most tolerant and those from Ramsey the least. The order of tolerance of animals from the other three other sites was Derbyhaven > Castletown > Peel.

The median lethal times (LT_{50}s) were generally higher at 0 psu than 60 psu (Fig 5.2) and these confirm the order of tolerance indicated by the time-response curves. However, analysis of variance (ANOVA) did not show any significant difference in mean LT_{50}s between sites at any salinity exposure (see Table 5.1 and 5.2).

Fig. 5.3 shows that there were clear inter-site differences in the tolerance of *L. saxatilis* subjected to desiccation stress (aerial exposure at 24 ° C). Animals from Laxey which suffered the least mortalities at extreme salinities were amongst the most susceptible to aerial exposure, whereas Castletown samples showed the highest tolerance. Mean LT_{50} values for the various sites are presented in Fig 5.3 and these were shown by ANOVA to be significantly different ($p<0.0001$) (Table 5.3). Multiple

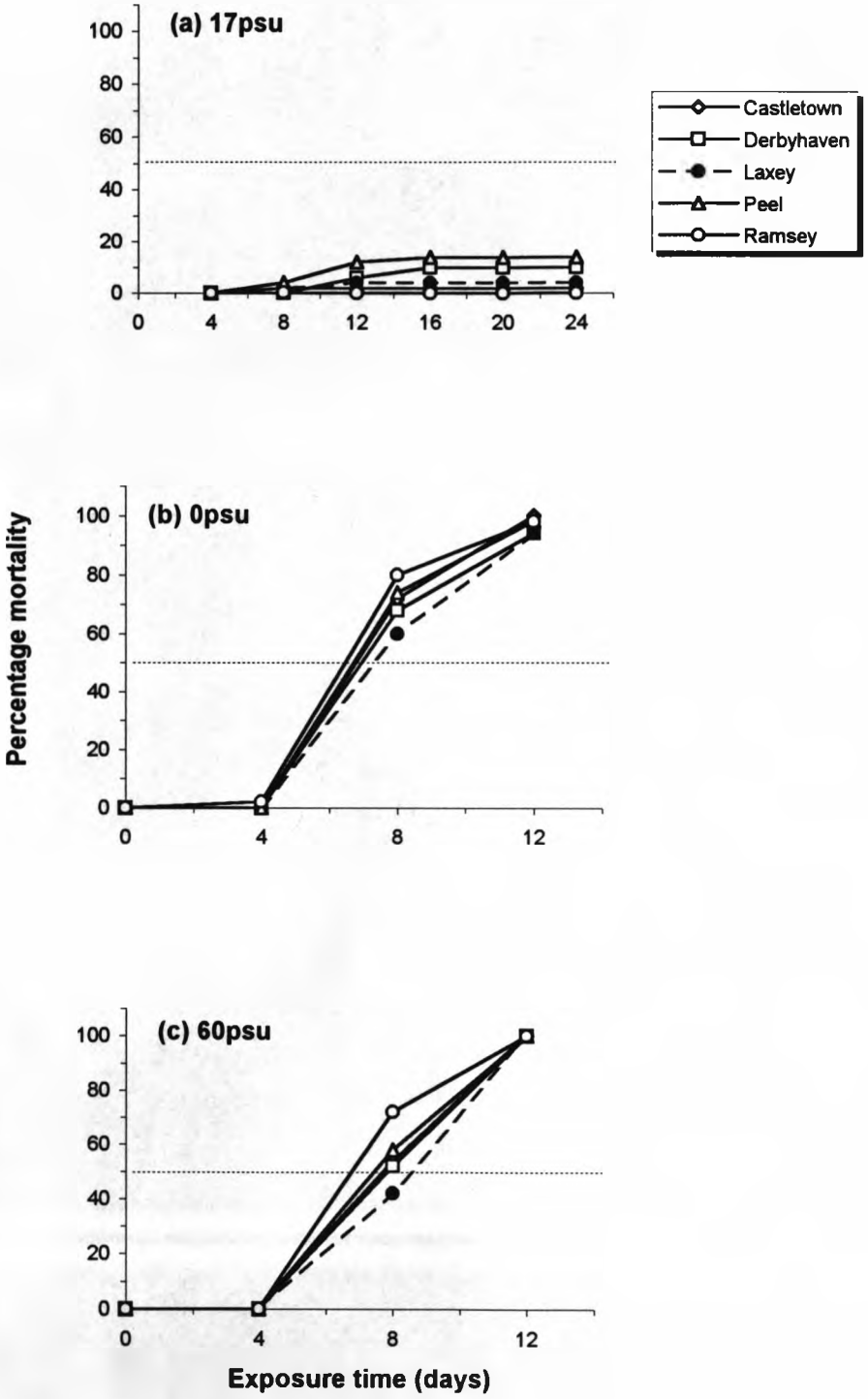

Fig. 5.1 Time-Mortality curves for *Littorina saxatilis* from five sites in the Isle of Man exposed to 0 psu, 17 psu and 60 psu salinity. Values plotted are means of five replicates (N.B. differences in exposure time). Dotted lines represent 50 percent mortality. Control mortality (0 %) not plotted.

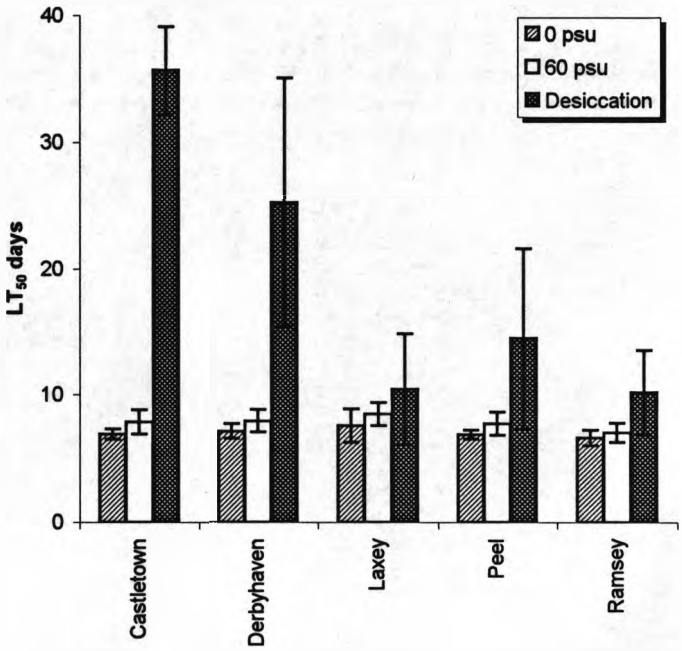

Fig. 5.2 Median Lethal Times (LT_{50}s) of *Littorina saxatilis* from five sites in the Isle of Man exposed to extreme salinity (0 psu and 60 psu) and desiccation (aerial exposure at 24 ° C) stress. Values plotted are mean ± s.d., n=5.

Table 5.1 Randomised-block Analysis of variance on LT_{50} values [log(x+1) transformed] of *Littorina saxatilis* from five sites in the Isle of Man exposed to 0 psu salinity.

Source of Variation	df	SS	MS	F	P-value	$F_{0.05}$
Site	4	0.0067	0.0017	2.978	0.051	3.007
Blocks	4	0.0192	0.0048			
Remainder	16	0.0090	0.0006			
Total	24	0.0349				

Table 5.2 Randomised-block Analysis of variance on LT_{50} values [log(x+1) transformed] of *Littorina saxatilis* from five sites in the Isle of Man exposed to 60 psu salinity.

Source of Variation	df	SS	MS	F	P-value	$F_{0.05}$
Site	4	0.0135	0.0034	1.749	0.189	3.007
Blocks	4	0.0062	0.0016			
Remainder	16	0.0309	0.0019			
Total	24	0.0507				

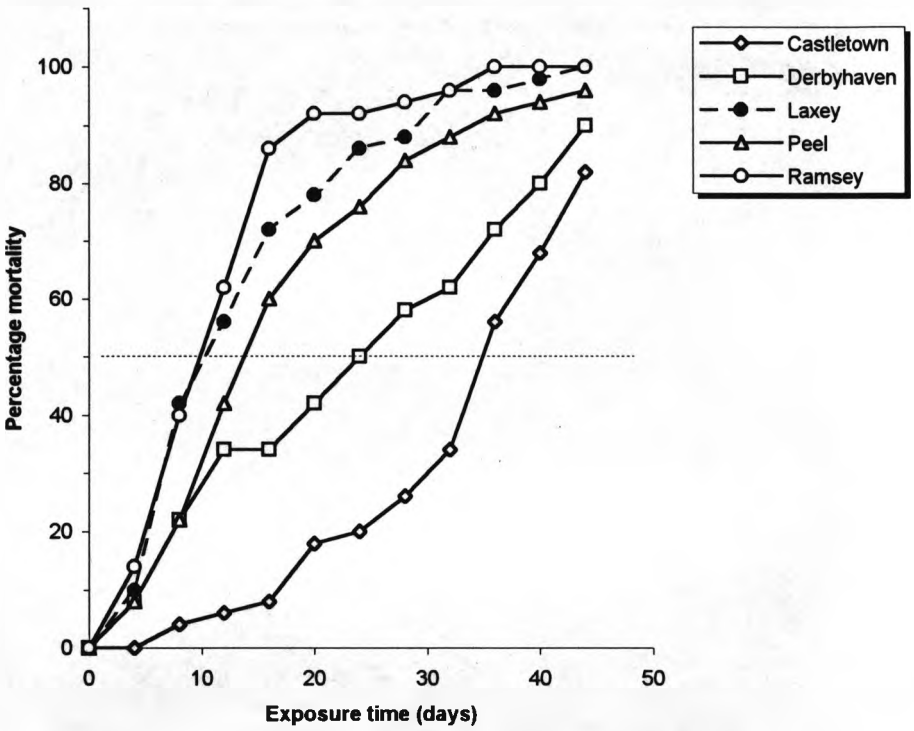

Fig. 5.3 Time-Mortality curves for *Littorina saxatilis* from five sites in the Isle of Man exposed to desiccation stress (aerial exposure at 24 ° C). Values plotted are means of five replicates. Dotted lines represent 50 percent mortality.

Table 5.3 One-way Analysis of variance on LT_{50} values [log(x+1) transformed] of *Littorina saxatilis* from five sites in the Isle of Man subjected to desiccation stress (aerial exposure at ca 24 °C).

Source of Variation	df	SS	MS	F	P-value	$F_{0.05}$
Site	4	1.1203	0.2801	13.489	0.0001	2.866
Error	20	0.4153	0.0208			
Total	24	1.5356				

Significance levels of Tukey HSD comparisons on mean LT_{50} values

	Castletown	Derbyhaven	Peel	Laxey
Castletown				
Derbyhaven	ns			
Peel	**	ns		
Laxey	***	**	ns	
Ramsey	***	**	ns	ns

*=p<0.05
**=p<0.01
***=p<0.001
ns=p>0.05

Table 5.4 Simple correlations (r) between LT_{50} values of *Littorina saxatilis* exposed to general stressors (salinity and aerial exposures) and LT_{50}s for exposure to different heavy metals. Asterisks indicate significant correlations (n=5).

Metals	Conc. (mgl^{-1})	Desiccation	0 psu	60 psu
Zinc	10	-0.403	0.881**	0.738
Zinc	20	0.528	0.772*	0.909**
Lead	5	-0.587	0.797*	0.552
Lead	10	0.091	0.614	0.441
Lead	20	0.308	0.838*	0.795*
Copper	0.5	0.321	0.822*	0.916**
Copper	1	0.125	0.701	0.848*
Copper	2	0.485	0.820*	0.915**
Cadmium	1	0.283	0.302	0.219
Cadmium	2	-0.459	0.331	0.002

Levels of significance
* p<0.05
** p<0.01
*** p<0.001
others p>0.05

comparisons by Tukey test indicate that animals from both Castletown and Derbyhaven are significantly more tolerant to desiccation than those from Laxey and Ramsey (see Table 5.3). Individuals from Castletown also had significantly higher LT_{50} values than those from Peel ($p<0.005$); but, no significant difference was found between the Derbyhaven and Castletown populations. *L. saxatilis* from all sites were less susceptible to desiccation stress than exposures to 0 psu and 60 psu salinities (see Fig 5.2).

The relationships between tolerance to generalised stress (aerial exposure, 0psu and 60 psu salinity) and metal (Zn, Pb, Cu and Cd) tolerance are presented as scatter plots of LT_{50}s at the various metal-concentration combinations against LT_{50}s of the general stressors (Figs 5.4-5.6). Correlation coefficients between LT_{50} values are presented in Table 5.4. It is interesting to note that there were negative correlations (although not significant) between desiccation :10mg l^{-1} Zn/5 mg l^{-1} Pb - the concentrations at which clear tolerance was demonstrated for these metals by animals from Laxey. There was also a non-significant negative correlation between desiccation and 2mg l^{-1} Cd. All correlations between 0 psu and 60 psu and all metal-concentration combinations were positive, and some were significant, but the correlations with Cd were low (see Table 5. 4).

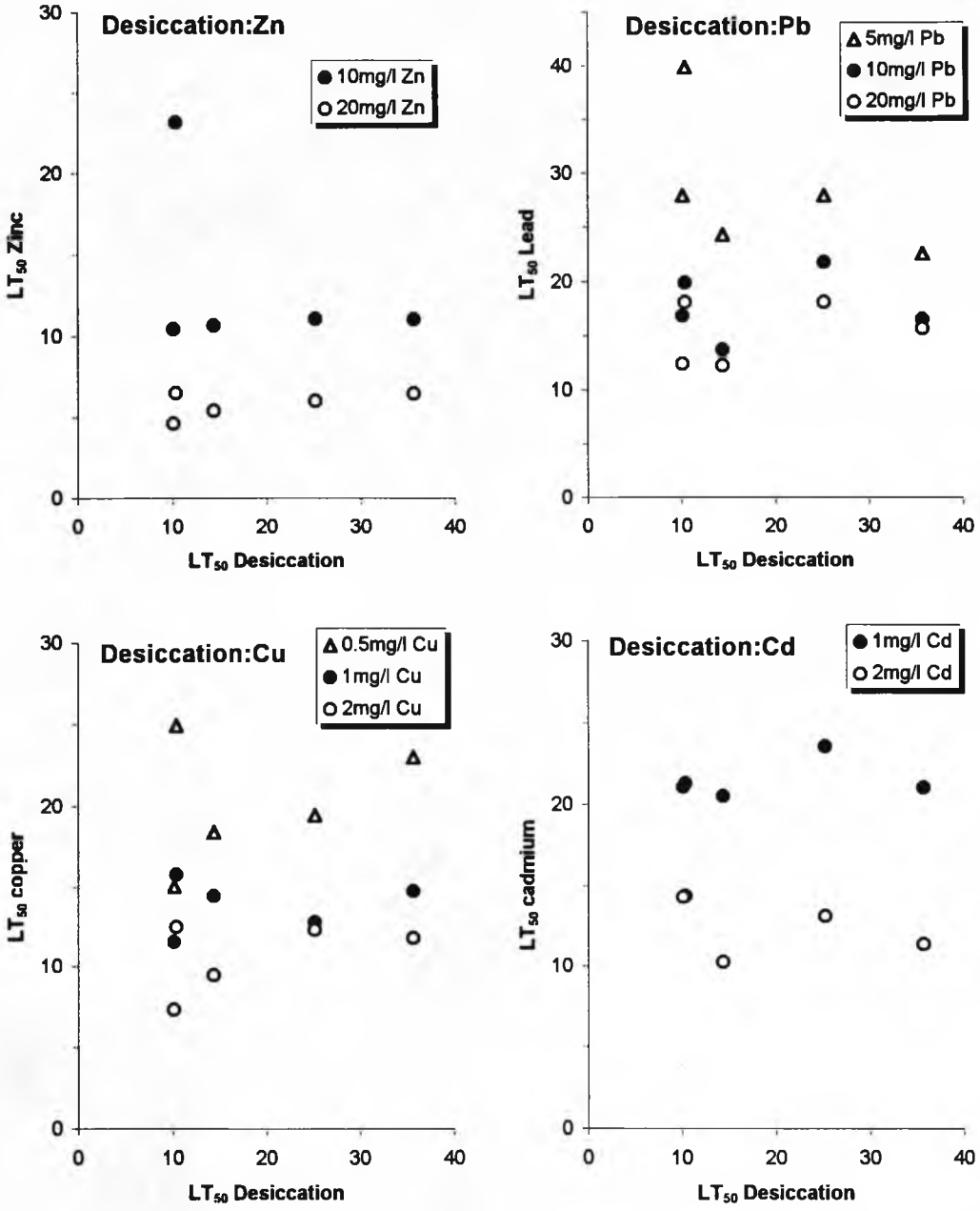

Fig 5.4 Scatter-plots of LT_{50} values of *Littorina saxatilis* exposed to desiccation stress against LT_{50} for tolerance to different heavy metal concentrations. Metal concentrations are 10 & 20 mgl^{-1} Zn; 5, 10, & 20 mgl^{-1} Pb; 0.5, 1 & 2 mgl^{-1} Cu; and, 1 & 2 mgl^{-1} Cd. See Table 5.4 for correlation coefficients.

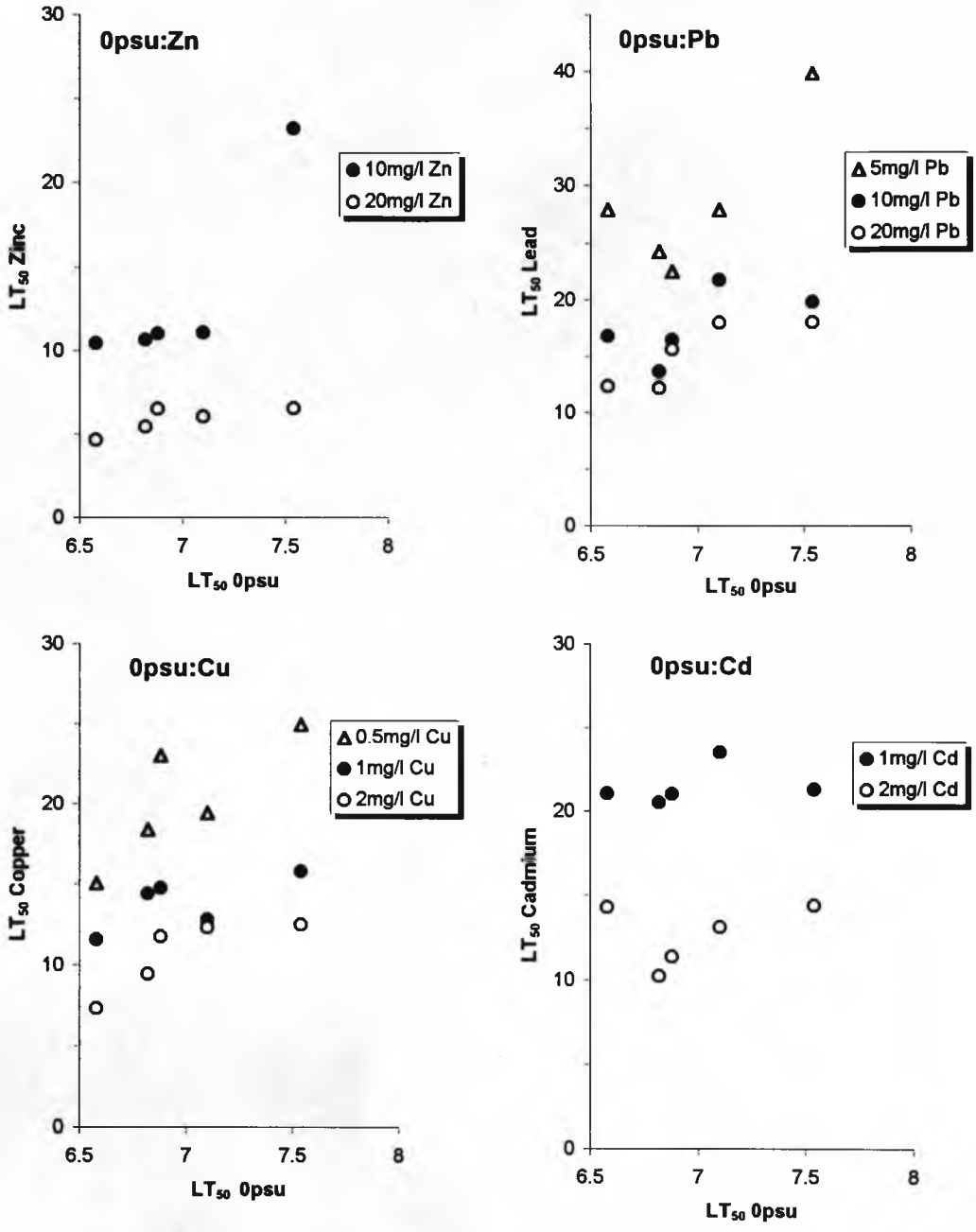

Fig 5.5 Scatter-plots of LT_{50} values of *Littorina saxatilis* exposed to 0 psu salinity against LT_{50} for tolerance to different heavy metal concentrations. Metal concentrations are 10 & 20 mgl^{-1} Zn; 5, 10, & 20 mgl^{-1} Pb; 0.5, 1 & 2 mgl^{-1} Cu; and, 1 & 2 mgl^{-1} Cd. See Table 5.4 for correlation coefficients.

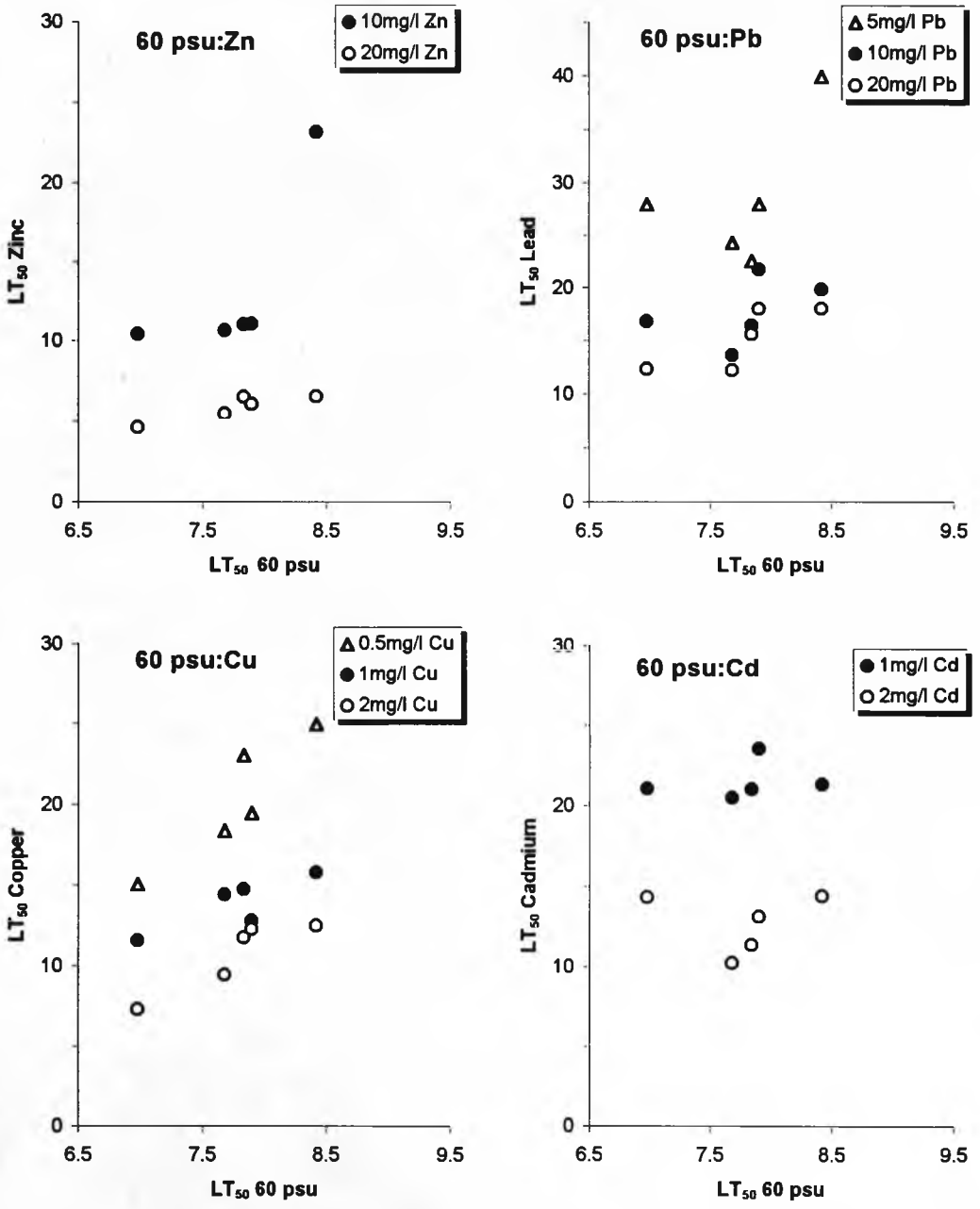

Fig 5.6 Scatter-plots of LT_{50} values of *Littorina saxatilis* exposed to 60 psu salinity against LT_{50} for tolerance to different heavy metal concentrations. Metal concentrations are 10 & 20 mgl^{-1} Zn; 5, 10, & 20 mgl^{-1} Pb; 0.5, 1 & 2 mgl^{-1} Cu; and, 1 & 2 mgl^{-1} Cd. See Table 5.4 for correlation coefficients.

5.4 DISCUSSION

No significant differences were found in tolerance to extreme salinity stress in *Littorina saxatilis* from sites in the Isle of Man. However, significant differences were obtained on exposure to desiccation stress, with animals from Laxey which showed enhanced tolerance to Zn and Pb being amongst the most susceptible to desiccation. In addressing the questions raised in the introduction, these results would lead to the conclusion that, in the first instance, the responses to both desiccation and salinity stress confirm that metal tolerance is not a mere expression of general fitness. Secondly, the detection of any physiological trade-off or "cost" of metal tolerance would depend on the stressor applied. The desiccation experiment detected an apparent "cost" of tolerance to metals as the LT_{50} values for desiccation were negatively correlated with LT_{50} values for exposure to 10mg l^{-1} Zn and 5mg l^{-1} Pb (the concentrations at which unequivocal tolerance was demonstrated for these metals by the animals from Laxey). Also, animals from the uncontaminated sites, Castletown and Derbyhaven, were significantly more tolerant to desiccation than those from Laxey. However, no significant differences in LT_{50} values were found between the winkles from Ramsey, Peel and Laxey. The pattern of intraspecific tolerance to extreme salinity exposure did not detect any physiological trade-off in animals from Laxey. In fact, animals from Laxey nominally showed the least susceptibilities at both salinity concentrations. However, these responses would have to be examined in the light of uncontrollable spatial heterogeneity of conditions at the sites from which the animals were collected.

Differences in salinity and emersion regimes, as well as exposure of habitat and shore height between the sites of collection could affect tolerance to salinity. Arnold (1972) observed differences in response to reduced salinity in littorinids from different sites. The lowest mortalities and highest activity scores were obtained at salinities approaching those prevailing at the site of collection. *L. saxatilis* from exposed shores in Sweden were found by Sundell (1985) to be more tolerant of reduced salinities than animals from sheltered habitats. Tolerance to low salinity has been found to be greatest in populations collected from sites subjected to reduced salinity. Rosenberg & Rosenberg (1973) reported that *L. littorea* in three Scandinavian populations showed varying tolerance to reduced salinity with the population from the area of lowest salinity showing the least susceptibility. Intraspecific differences in response to reduced salinity also occur with shore height (Arnold, 1972). The animals from Laxey are found further up the estuary than those from all other sites and are likely to experience higher salinity variations. It was therefore not surprising that animals from Laxey would be more tolerant than those from other sites. It is difficult to assume that they would have had a significantly higher tolerance if they were not exposed to metal stress.

The manner in which physiological modifications occur in the animals in response to salinity and aerial exposure may also explain the differences in response to the two stressors. Marine molluscs are generally considered to be poikilosmotic animals with no power of extracellular osmotic regulation (Hoyaux *et al.*, 1976) and such is the case with littorinids (Mayes, 1962, Avens & Sleigh, 1965, Taylor & Andrews, 1988). However, behavioural responses, notably shell closure may enable animals in some cases to temporarily isolate themselves from the external medium and reduce the

amplitude of osmotic shock. Avens & Sleigh (1965) observed that *L. saxatilis* maintained a hyperosmotic state for up to 3 days in low salinity solutions using a "shell closing" mechanism. They also found that on exposure to low salinity, *L. saxatilis* does not behave as a perfect osmometer, but rather as a leaky one allowing some ionic movements. On transfer from 100 % to 50 % seawater, an inflow of water resulting in a rapid increase in weight was accompanied by a loss of salts, but the final weight at 50 % seawater reached a steady level above that in 100 % seawater. Mayes (1962) similarly found about 10 % gain in wet tissue weight after 36 hrs of transfer of *L. saxatilis* from 100 % to 50 % seawater. Immersion in hypersaline water (200 % seawater), on the contrary, resulted in a decrease in weight.

Up to 45 % loss of weight was recorded after a week of aerial exposure of *L. saxatilis* at 10-15 °C; and exposure for up to 12 days (beyond which no animals survived) resulted in about 100 % increase in blood urine concentration (Avens & Sleigh, 1965).

There is considerable difference in the survival capacity of *L. saxatilis* to extreme salinity and aerial exposures reported by different authors. The median lethal times obtained in this study (6.5-7.5 days) for exposure of *L. saxatilis* to 0 psu salinity are comparable with those of Cannon & Hughes (1992) for animals collected from Cable Bay, Anglesey (ca 5 days). Sundell (1985) reported a mean survival time of 14 to 18 days for animals from the Swedish coast. My results also differ considerably from those of Webb (1990) for animals from sites in the Isle of Man. Firstly, he found that animals from Castletown were much more tolerant at 0 psu salinity than those from Laxey with LT_{50} of ca. 10 and 5 days respectively. My values were ca. 7 days for Castletown and 7.5 days for Laxey. Secondly at 50 % seawater, I found minimal

mortality in animals from all sites over a 24-day exposure period while Webb (1990) found LT_{50} values of ca. 14 days in animals from Laxey and 10 days in those from Peel, but for individuals from Castletown <50 % mortality was obtained at the termination of his experiments after 20 days.

Euryhalinity in littorinids has been shown to increase with an increase in shore height above mean water mark (Walton, 1915; Mayes, 1962; Avens & Sleigh, 1965). An increase in uric acid production in those species of littorinids living higher up the shore compared to those lower down (Needham, 1938; Potts, 1967) tends to support this. However, Eichelberg & Heil (1989) argue that the formation of considerable amounts of uric acid in *Littorina littorea* may not be a means of excreting toxic nitrogenous wastes. The reason being that accumulated uric acid will not be excreted directly but after degradation to ammonia. Emmerson (1969) and Taylor & Andrews (1988) did not find any appreciable modification of ammonia excretion by osmotic stress in *L. sitchana* and *L. littorea* respectively. Smith (1994) reported that *L. saxatilis* collected from a boulder field low in the intertidal had a significantly higher uric acid concentration than those from a nearby cliff that were at the upper limit of their range. Smith concluded that although the crevice-dwelling animals are higher in the intertidal zone, they are in fact less subject to desiccation and the higher uric acid concentration of boulder field snails reflects a water conservation mechanism that is not required in the crevices.

The nature of experimental treatments may affect the response of animals to stress. When animals previously exposed to hyposaline and hypersaline solutions were re-introduced into 100 % seawater, a reversal in the weight changes resulted (Mayes,

1962; Avens & Sleigh, 1965). Similarly, a rapid rehydration and weight gain ensue when animals exposed to desiccation are immersed in seawater (Avens & Sleigh, 1965). The short periods of immersion in 100 % seawater during the examination of individuals for mortality may have resulted in increased osmotic shock in my salinity experiments. This may partly account for the huge mortalities obtained on day-eight (second examination) while no mortalities were obtained on day-four in both 0 psu and 60 psu exposures. However, for the desiccation experiment, the rehydration achieved during examination may have resulted in longer survival times. Avens & Sleigh (1965) recorded total mortalities in *L. saxatilis* within 12 days in experiments where animals were exposed to desiccation in jars and mortality examined by prodding. In contrast the animals from the least tolerant site in my study did not record 100 % mortality until day-36.

In conclusion, it appears that metal tolerance may be exerting an effect on the ability of the population of *L. saxatilis* from Laxey to withstand basic intertidal stress, especially desiccation. This is probably a physiological trade-off in function but ecological differences between sites may partly account for the differences observed.

CHAPTER SIX

ACCUMULATION OF HEAVY METALS IN *LITTORINA SAXATILIS* FROM A METAL CONTAMINATED AND UNCONTAMINATED SITES IN THE ISLE OF MAN

6.1 INTRODUCTION

Tolerance to acute levels of Zn and Pb has been demonstrated in *Littorina saxatilis* from a site (Laxey Estuary) contaminated with Zn and to some extent Pb, in the Isle of Man. The exhibition of such tolerance must result from the presence of mechanisms that enable tolerant populations to cope better with elevated metal levels. At least three mechanisms of metal tolerance exist in aquatic invertebrates: (i) reduced uptake (Bryan & Gibbs, 1983) (ii) storage in inert form which may be coupled with enhanced uptake (Bryan, 1976b; Brown, 1978) (iii) increased excretion (Robinson, 1985, Webb, 1990) (see Bryan, 1979 for general reviews). These traits may be developed to varying degrees, either singly or in combination to confer tolerance (Deplege, 1990)

Several workers have reported tolerance to heavy metals to be coincidental with lowered permeability to metals in aquatic organisms. Zn tolerant populations of the polychaete *Nereis diversicolor*, and the decapod crustacean *Carcinus maenas,* from Restronguet Creek have been reported to show reduced permeability to Zn (Bryan, 1976b, Bryan & Gibbs, 1983). Also, littorinids from Dulas Bay have been shown to acquire tolerance to Zn and Cu by taking up relatively less metal than animals from nearby control sites. This was shown for *Littorina obtusata* by Credland (1988) and for *L. saxatilis* by Webb (1990). In addition Webb noted a relatively higher efflux of Zn in the tolerant animals. Naylor (1987) found that Cu tolerance in *Cerastoderma edule* from the Tamar estuary was due to a slower accumulation rate in comparison with animals from an uncontaminated site; but excretion rates were similar.

Viarengo (1989) suggests that mechanisms of cellular compartmentalisation may play a fundamental role in reducing the cytotoxic effect of metals. Storage of metals in non-toxic forms may involve changes in metabolically active sites by binding to proteins (such as metallothioneins), incorporation of toxic metals in shell or exoskeleton, or by the manufacture of granules (Deplege, 1990). Metal containing granules have been found in a wide range of aquatic invertebrates (see Brown, 1982 for reviews). The existence of such granules in littorinids from metal contaminated sites has been reported (e.g. Mason & Nott, 1981, Mason *et al.*, 1984, Brough & White, 1990). In *L. saxatilis* the granules vary in size and appearance, particularly between tissues, but there appear to be two broad types (Brough & White, 1990). The first type is mineralized, with metal(s) probably associated with inorganic anions such as phosphate. These granules are inorganic, very hard and refractile, and typically contain Ca as well as heavy metals such as Zn and Mn. The second type of granule may be organic and rich in S, the metals largely complexed to proteins.

The role of metallothioneins and metallothionein-like proteins in the regulation and detoxification of metals has received considerable attention. The term "metallothionein" was used initially to designate the Cd-, Zn- and Cu- containing sulphur-rich protein from equine renal cortex (Kagi & Vallee, 1960, Fowler *et al.*, 1987). Metallothioneins have since been shown to be widely distributed, occurring in prokaryotes, protists, fungi, plants and animals and this has been reviewed by Roesijadi (1992). Increased tolerance to heavy metals associated with metallothioneins have been found in fish (e.g. Kito *et al.*, 1982; McCarter & Roch, 1983; Klaverkamp & Duncan, 1987) and aquatic invertebrates (e.g. Roesijadi, 1982; Roesijadi & Fellingham, 1987). Prior exposure to metals known to induce metallothioneins does

not always result in metal-tolerant individuals. Harrison *et al.* (1987) found that, although pre-exposure of the slipper limpet *Crepidula fornicata* to low concentrations of mercury in the laboratory resulted in enhanced binding mercury to metallothionein, no increased tolerance to mercury occurred. Metallothionein-like proteins have been reported from littorinids but these are not always associated with high metal levels (Howard & Nickless, 1978, Noel-Lambot *et al.*, 1978, Langston & Zhou, 1987).

The type of changes that are involved in the above processes may lead to different net metal burdens in tolerant and non-tolerant populations when they are exposed to similar metal levels. Differences in metal handling abilities in a metal tolerant and non-tolerant populations of *L. saxatilis* from sites in the Isle of Man were assessed in metal accumulation studies. Inter-site differences in the net uptake of Zn, Pb, Cu and Cd were examined. Also the effects of Pb, Cu and Cd on Zn accumulation (and the reciprocal effect of Zn on the accumulation of the three metals) were assessed.

6.2 MATERIALS AND METHODS

Three series of metal accumulation experiments were conducted. The first series started in June 1995 was for accumulation of Zn and Cu with time at fixed concentrations of the respective metals. The second series was for the accumulation of Cu, Cd and Pb with increased concentration in a fixed time. The third assessed the effects of Cd, Cu and Pb on Zn accumulation. All experiments were performed with stable metal as radiolabelling facilities were not available.

6.2.1 Zn and Cu accumulation with time

Test animals were collected from Castletown, Derbyhaven, Laxey, Peel and Ramsey in June 1995 and acclimatised to laboratory conditions as in chapter 4.2. Animals were counted into bags of thirty for further acclimatisation to test chambers. Before exposure, four individuals were removed from each bag for assessment of initial metal content. The rest were transferred into three replicates of the test concentrations of 2.5 and 5 mg l^{-1} added Zn, and 0.5 mg l^{-1} added Cu. Four individuals representing a pooled sample for metal analysis were collected from each bag every two days and this was continued till the 8th day. All samples were analysed for the respective metals by atomic absorption spectrophotometery as described in chapter 3.2. The regressions of metal concentration against time were plotted and the slopes of the regressions compared by Analysis of Covariance (ANCOVA) using MINITAB for Windows version 10. For the Zn accumulation experiments, the results for day-8 were not included due to accidental loss of samples.

6.2.2 Metal accumulation with increased concentration in a fixed time

For Pb and Cd, this approach was adopted because of generally low initial levels in *L. saxatilis* (see 3.3), such that four pooled individuals may not give accurate results. For Cu this was done to check how Cu accumulation would compare with what was obtained from the above method. The experiments were conducted in two batches; the first in July-August 1995 for Pb and Cu accumulation and then in late August 1995 for Cd accumulation.

Animals were obtained from the above sites and acclimatised. Matched sets of 10 individuals per bag were exposed in triplicate to the appropriate metal concentrations after acclimatisation to the test chambers. The concentrations were 0.05, 0.1, 0.5 and 1 mg l^{-1} added Cu and added Cd; 0.1, 0.5, 1 and 2 mg l^{-1} added Pb and added Zn. The solutions were changed every 2 days and the experiments were terminated after 6 days. At the end of each exposure to metals, the animals were boiled for a few seconds and stored frozen until analysed for the respective metals.

6.2.3 Effect of Cd, Cu and Pb on Zn accumulation

Animals collected in September 1995 and acclimatised as above in bags of 10 were used to test the effects of the three metals on Zn accumulation. Animals were exposed in triplicate treatments to 2 mg l^{-1} Zn alone or a mixture of 2 mg l^{-1} Zn and 0.01, 0.1, 1 mg l^{-1} added Cu, Cd, or Pb. The exposure was terminated after 6 days and the

solutions were changed every other day. Samples of 8 pooled individuals per treatment were analysed for the Zn and the respective metals.

6.3 RESULTS

6.3.1 Zn, Pb, Cu and Cd accumulation in individual metal exposure

Accumulation of Zn in *L. saxatilis* from five sites at 2.5 mg l^{-1} and 5 mg l^{-1} added Zn are given as log-log regressions of tissue metal concentrations against time in Figs. 6.1 and 6.2. The estimated regression equations and statistics are presented in Table 6.1. At both concentrations, individual regression lines were significant for samples from all sites. The slopes of the regressions indicate that the animals from Laxey had a much lower rate of Zn accumulation than individuals from all other sites. Analysis of Covariance (ANCOVA) showed that there was a significant difference in regression slopes between sites for each Zn exposure concentration (Table 6.2).

Fig 6.3 shows the regressions of Pb accumulation with increasing Pb concentration. All individual regression lines were highly significant ($P<0.0001$) (Table 6.3). ANCOVA showed a significant difference between slopes ($p<0.01$, Table 6.4), implying that there were different rates of Pb accumulation between sites. *L. saxatilis* from Laxey had the highest slopes, and animals from other sites were in the order Peel > Castletown > Ramsey > Derbyhaven (see Table 6.3).

Cu accumulation (at 0.5mg l^{-1} Cu) with time gave significant regressions in animals from all five sites (Fig. 6.4, Table 6.5). There were minimal differences in regression slopes between sites (Table 6.5) and these were confirmed by ANCOVA to be non-significant (Table 6.6). The regression coefficients (r^2) for Cu accumulation with time were rather low, ranging from 49.2 % for animals from Ramsey and 80.7 % for those from Laxey. A second experiment performed to examine Cu accumulation with

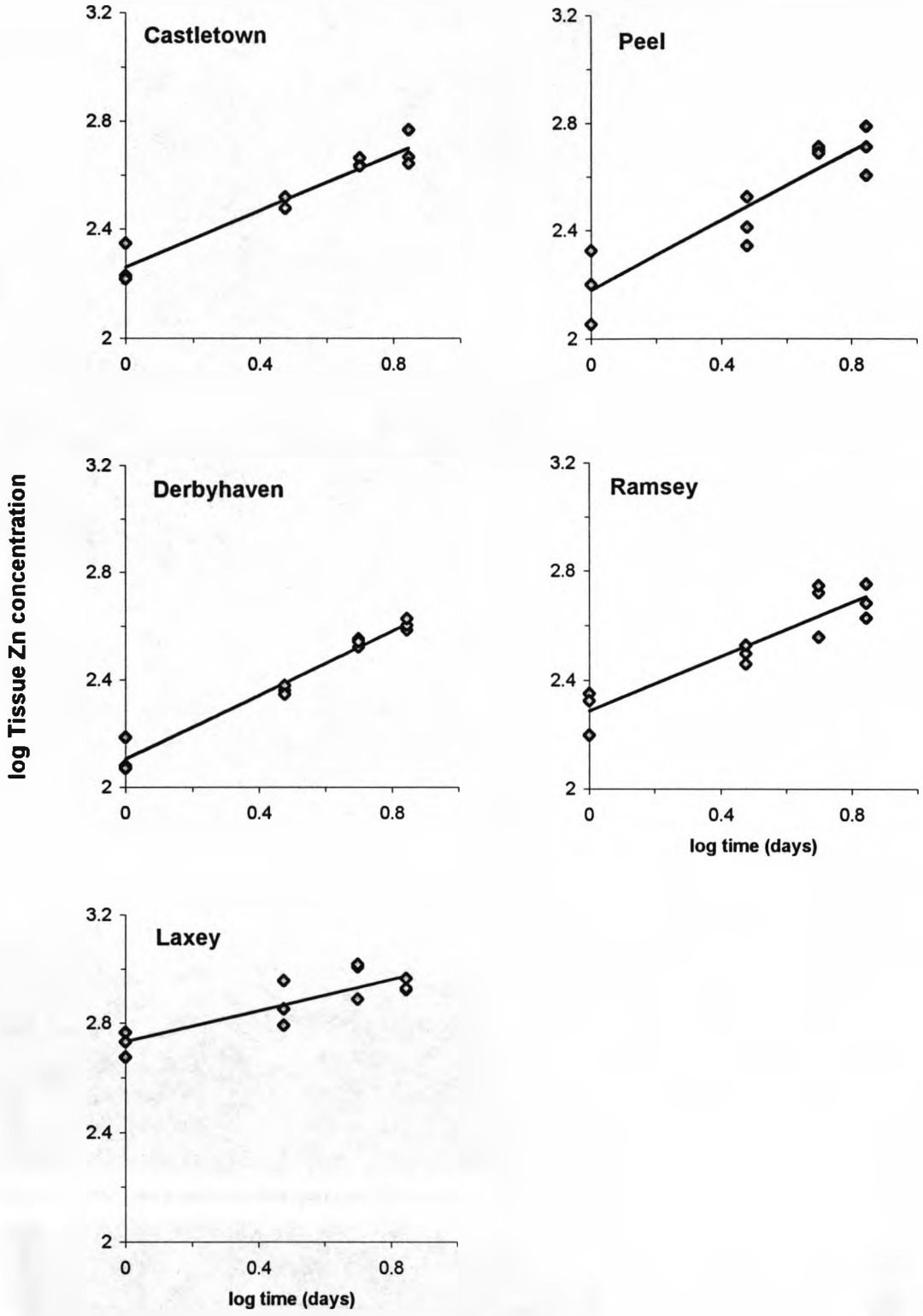

Fig 6.1 Regressions of zinc accumulation with time in *Littorina saxatilis* from five sites in the Isle of Man exposed to 2.5 mgl^{-1} added Zn. Tissue Zn concentrations (μgg^{-1} dry weight) and exposure time (days) were log(x+1) transformed. See Table 6.1a for regression equations.

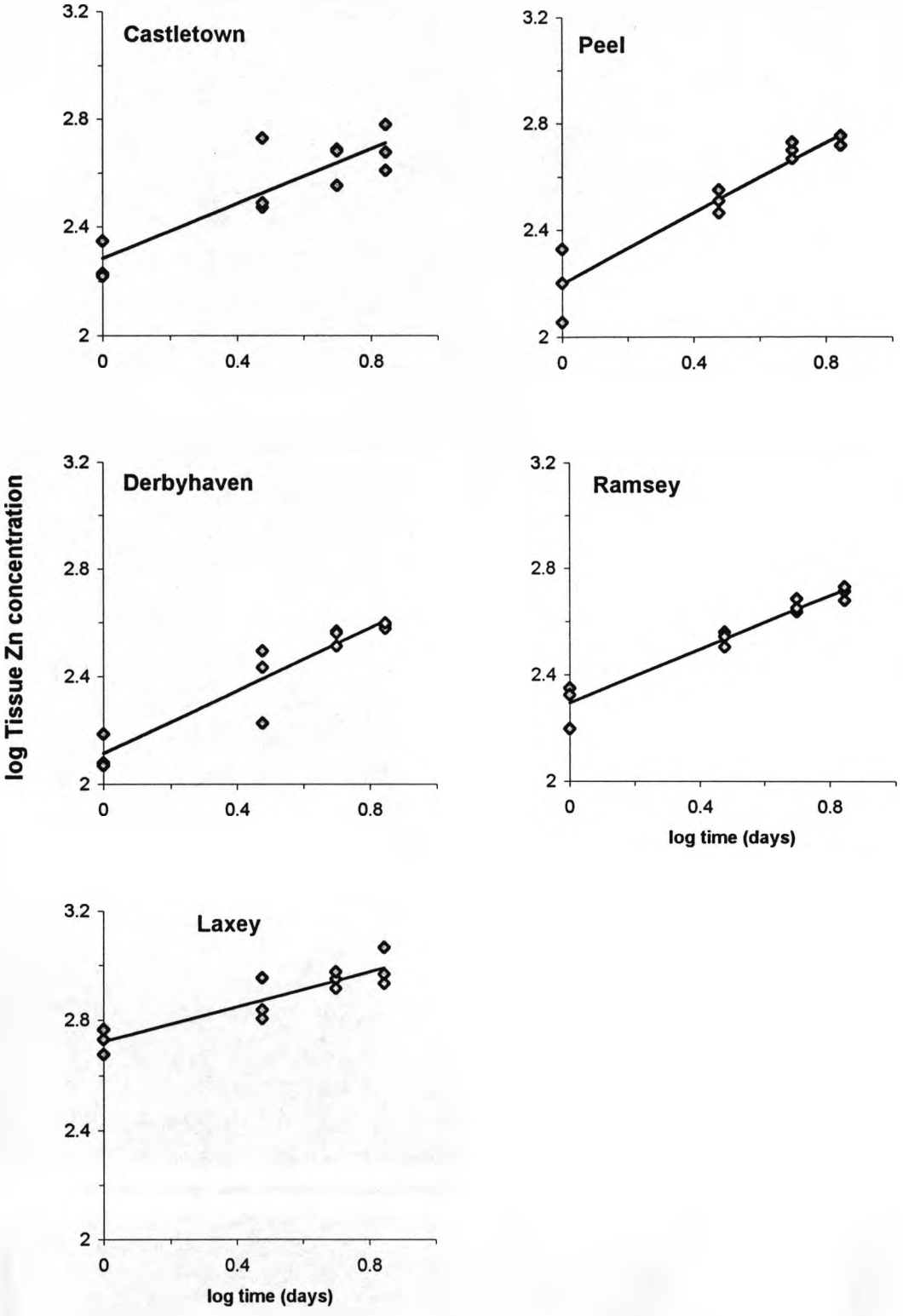

Fig 6.2 Regressions of zinc accumulation with time in *Littorina saxatilis* from five sites in the Isle of Man exposed to 5 mgl^{-1} added Zn.Tissue Zn Concentration (μgg^{-1} dry weight) and exposure time (days) were log(x+1) transformed. See Table 6.1b for regression equations.

Table 6.1 Regression equations and statistics for the accumulation of zinc from solution (2.5 and 5 mgl^{-1} added Zn) in *Littorina saxatilis* from five sites in the Isle of Man.

Site	n	Equation	r^2 (%)	F-value	p<
(a) 2.5 mgl^{-1} Zn		a b			
Castletown	12	logy = 2.26 + 0.519 logx	93.5	143.71	0.0001
Derbyhaven	12	logy = 2.11 + 0.593 logx	97.0	321.82	0.0001
Laxey	12	logy = 2.73 + 0.285 logx	72.6	26.45	0.0001
Peel	12	logy = 2.18 + 0.644 logx	83.7	51.23	0.0001
Ramsey	12	logy = 2.29 + 0.496 logx	85.1	57.23	0.0001
(b) 5 mgl^{-1} Zn					
Castletown	12	logy = 2.28 + 0.508 logx	78.8	37.13	0.0001
Derbyhaven	12	logy = 2.11 + 0.582 logx	88.9	79.72	0.0001
Laxey	12	logy = 2.72 + 0.316 logx	81.8	44.85	0.0001
Peel	12	logy = 2.20 + 0.662 logx	91.4	105.67	0.0001
Ramsey	12	logy = 2.29 + 0.502 logx	94.3	165.69	0.0001

y = tissue Zn concentration, x = days

Table 6.2 Analysis of Covariance (ANCOVA) to test for significant differences in zinc accumulation in *Littorina saxatilis* from five sites in the Isle of Man exposed to 2.5 mgl^{-1} and 5 mgl^{-1} added zinc over a period of 6 days. Tissue metal values and exposure time were log(x+1) transformed. Separate analysis performed for each concentration.

(a) ANCOVA for Zn accumulation at 2.5mgl^{-1} added Zn

Source of Variation	df	SeqSS	AdjSS	AdjMS	F	P-value
Days	1	0.22425	0.22425	0.22425	5.76	<0.001
Site	4	0.13607	0.70888	0.17722	4.55	<0.001
SitexDay	4	1.12943	1.12943	0.28236	7.25	<0.001
Error	50	1.94755	1.94755	0.03896		
Total	59	3.43731				

(b) ANCOVA for Zn accumulation at 5mgl^{-1} added Zn

Source of Variation	df	SeqSS	AdjSS	AdjMS	F	P-value
Days	1	0.23952	0.23952	0.23952	6.20	<0.05
Site	4	0.15418	0.73787	0.18447	4.78	<0.01
SitexDay	4	1.11778	1.11778	0.27945	7.24	<0.001
Error	50	1.93056	1.93056	0.03861		
Total	59	3.44204				

N.B. A significant interaction between days and site indicates a significant difference between regression slopes.

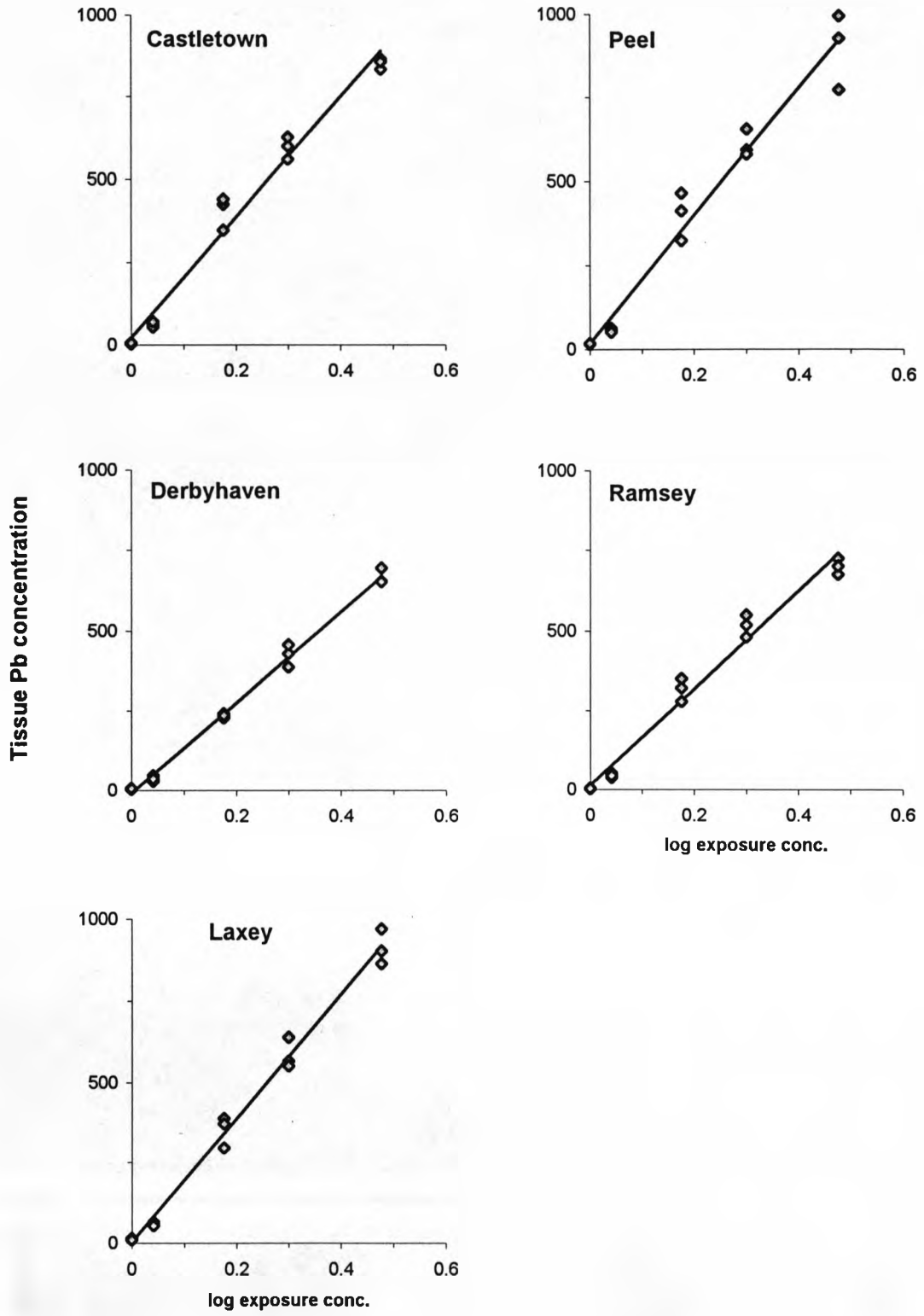

Fig 6.3 Regressions of tissue lead burdens ($\mu g g^{-1}$ dry weight) with increasing exposure concentration (mgl^{-1} Pb) in *Littorina saxatilis* from five sites in the Isle of Man. Exposure conc. log (x+1) transformed. Animals were exposed to different lead concentrations for six days. See Table 6.3 for regression equations.

Table 6.3 Regression equations and statistics for the accumulation of lead from solution in *Littorina saxatilis* from five sites in the Isle of Man.

Site	n	Equation	r^2 (%)	F-value	p<
		a b			
Castletown	15	y = 21.8 + 1817 logx	98.0	652.2	0.0001
Derbyhaven	15	y = 8.34 + 1416 logx	99.5	2385.6	0.0001
Laxey	15	y = 3.10 + 1917 logx	99.0	1265.9	0.0001
Peel	15	y = 17.5 + 1901 logx	96.8	398.4	0.0001
Ramsey	15	y = 13.2 + 1521 logx	97.8	582.4	0.0001

y = tissue lead concentration; x = lead concentration in solution

Table 6.4 Analysis of Covariance (ANCOVA) to test for significant differences in lead accumulation in *Littorina saxatilis* from five sites in the Isle of Man exposed to a range of lead concentrations for six days. Exposure concentrations were log(x+1) transformed.

Source of Variation	df	SeqSS	AdjSS	AdjMS	F	P-value
Concentration	1	6731935	6731935	6731935	3363.5	<0.001
Site	4	171280	3835	959	0.48	0.751
SitexConcentration	4	97309	97309	24327	12.15	<0.001
Error	65	130094	130094	2001		
Total	74	7130619				

N.B. A significant interaction between site and concentration indicates a significant difference between regression slopes.

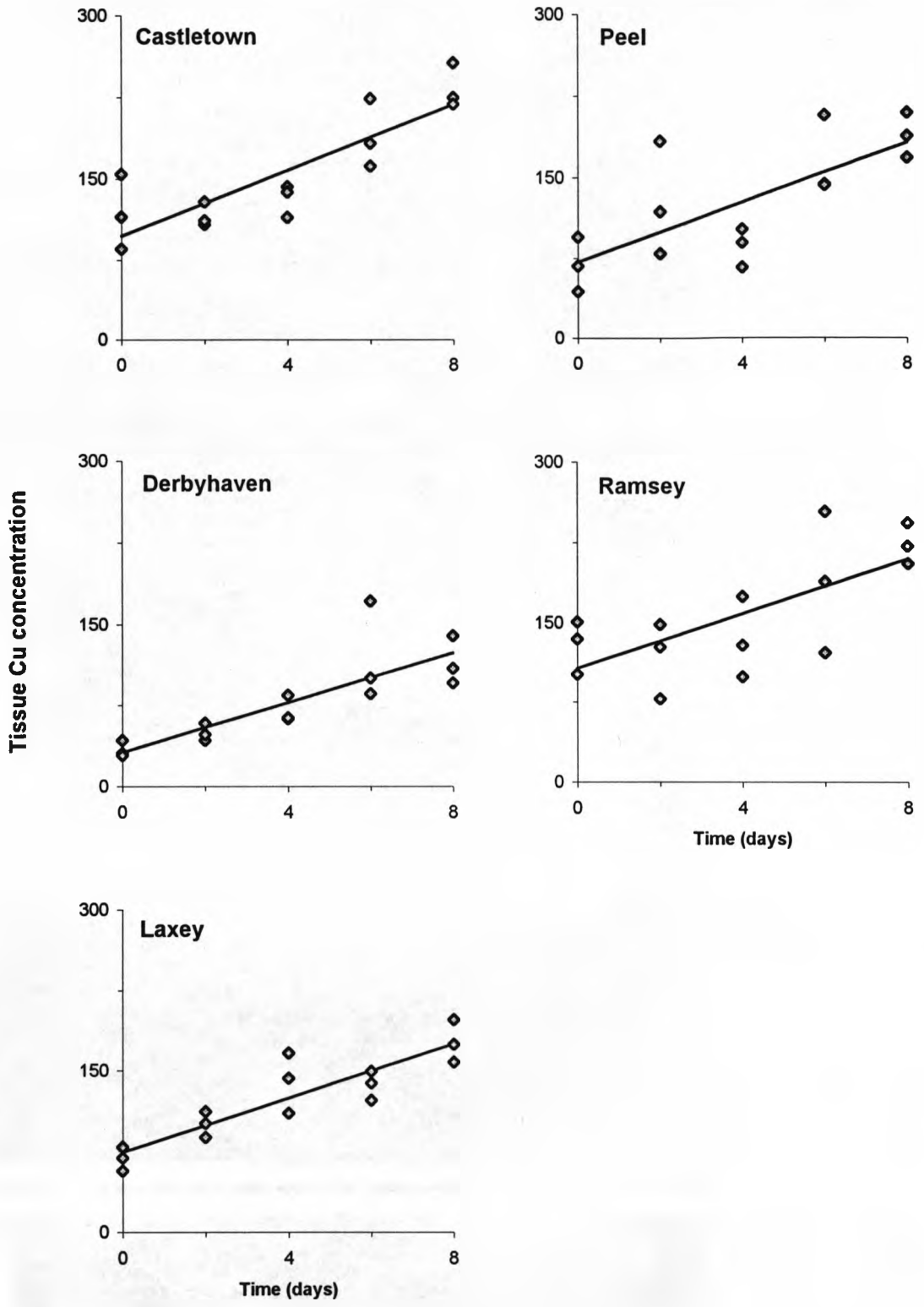

Fig 6.4 Regressions of copper accumulation with time in *Littorina saxatilis* from five sites in the Isle of Man exposed to 0.5 mgl^{-1} added Cu. Tissue Cu concentrations in μgg^{-1} dry weight. See Table 6.5 for regression equations.

Table 6.5 Regression equations and statistics for the accumulation of copper with time (from solutions of 0.5 mgl^{-1} added Cu) in *Littorina saxatilis* from five sites in the Isle of Man.

Site	n	Equation	r^2 (%)	F-value	p<
		a b			
Castletown	15	y = 97.0 + 15.2x	72.8	34.8	0.0001
Derbyhaven	15	y = 31.5 + 11.5x	68.5	28.3	0.0001
Laxey	15	y = 73.6 + 12.7x	80.7	54.5	0.0001
Peel	15	y = 71.7 + 13.9x	55.1	15.9	0.01
Ramsey	15	y =107.0 + 12.9x	49.2	12.6	0.01

y = tissue copper concentration; x = days

Table 6.6 Analysis of Covariance (ANCOVA) to test for significant differences in copper accumulation in *Littorina saxatilis* from five sites in the Isle of Man exposed to 0.5 mgl^{-1} added Cu over a period of eight days.

Source of Variation	df	SeqSS	AdjSS	AdjMS	F	P-value
Days	1	105174	105174	105174	110.6	<0.001
Site	4	65336	16944	4236	4.45	<0.01
SitexDay	4	905	905	226	0.24	0.916
Error	65	61813	61813	951		
Total	74	233228				

See note on Table 6.2

increasing Cu concentration over a fixed period gave interesting net uptake curves (see Figs. 6.5 and 6.6). At the lower concentrations (0.1 to 0.5mg l^{-1} Cu), Cu accumulation increased with concentration. Above 0.5 mg l^{-1} a sharp decline in net uptake occurred in animals from all sites. Linear curve fits with the tissue Cu levels for 1 mg l^{-1} Cu exposure included were poor with very low regression coefficients (Fig 6.5 and Table 6.7a). The exclusion of the values for 1.0 mgl^{-1} Cu improved the linearity of the fit lines (Fig 6.6 and Table 6.7b). However, ANCOVA on the regressions with all values included or without the values for 1.0 mgl^{-1} showed no significant difference in slopes between sites (Table 6.8a & b).

Regression lines for the net uptake of Cd with increasing Cd concentration are given in Fig. 6.7. Individual regressions were significant for animals from all the five sites ($p<0.0001$). The slopes varied with site and individuals from Peel had the highest, whilst animals from Derbyhaven had the least (Table 6.9). The differences in slope were significant (ANCOVA, Table 6.10).

6.3.2 Effects of Cu, Cd and Pb on Zn accumulation

The effects of increasing concentrations of Cu, Cd and Pb (0.01, 0.1, and 1 mg l^{-1} added metal) on Zn accumulation (at constant Zn concentration, 2 mg l^{-1}) are presented in Figs 6.8 to 6.10. Of the three metals, Cu was found to have the most profound effect on Zn accumulation (Fig 6.8). An increase in Zn accumulation (above values observed in Zn only solutions) was observed in mixtures with the lower concentrations of Cu, with values at 0.1mgl^{-1}Cu being higher than values for 0.01mgl^{-1}.

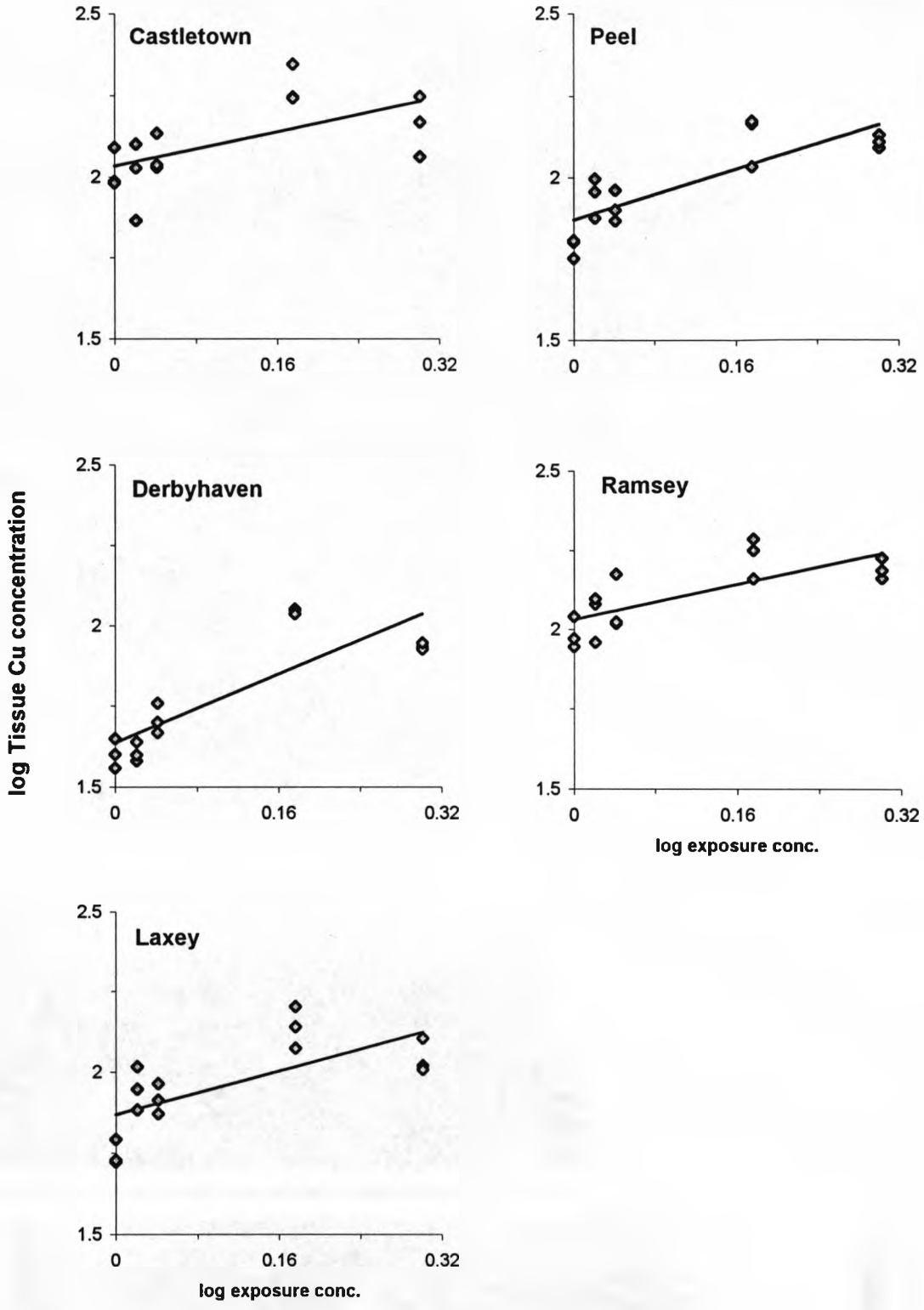

Fig 6.5 Regressions of tissue copper burdens (μgg^{-1} dry weight) with increasing exposure concentration (mgl^{-1} Cu) in *Littorina saxatilis* from five sites in the Isle of Man. Tissue Cu concentration and exposure conc. were log(x+1) transformed. Animals were exposed to different Cu concentrations for six days. See regression equations in Table 6.7a.

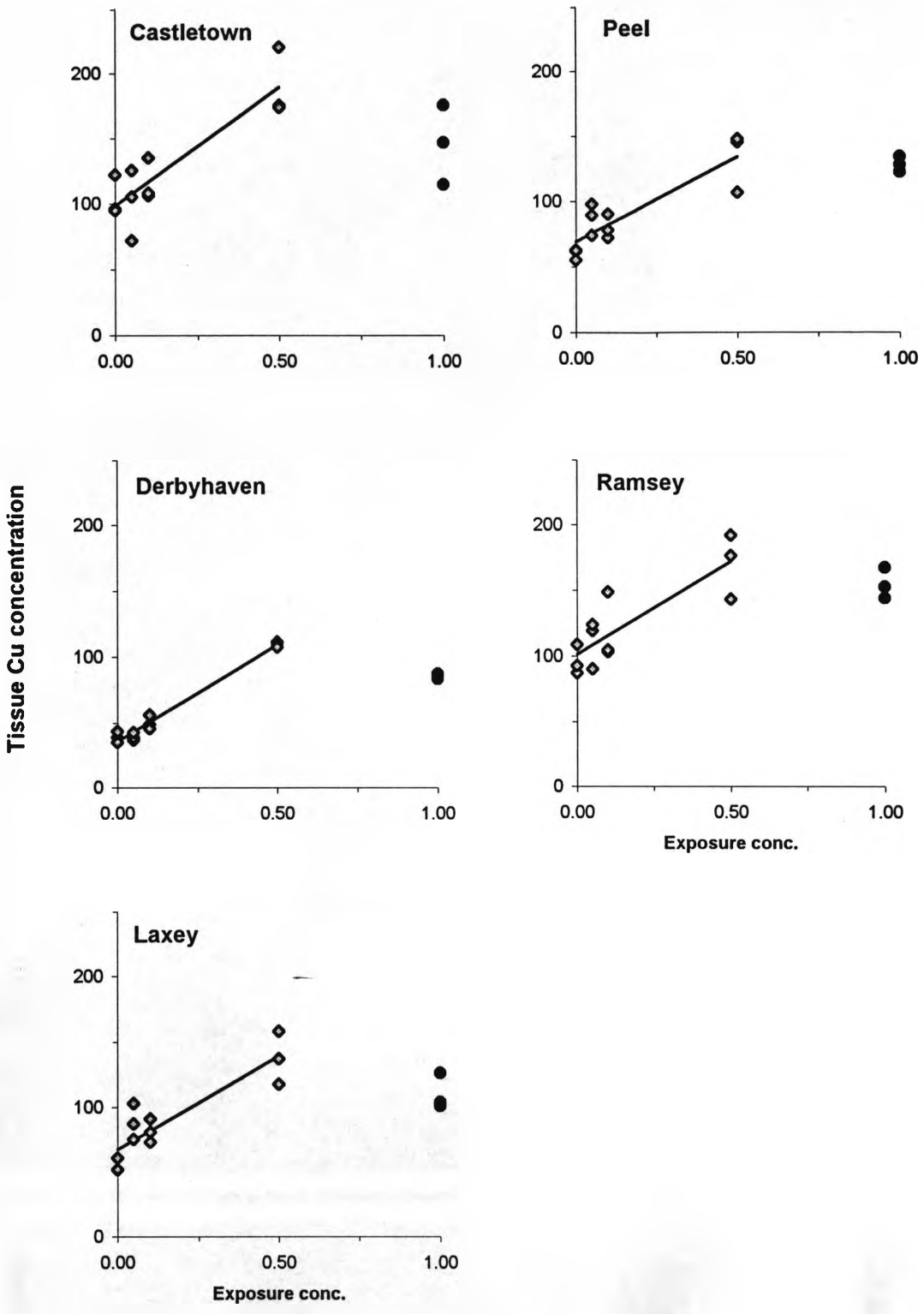

Fig 6.6 Regressions of copper accumulation in Fig. 6.5 re-fitted without values for 1.0 mgl^{-1} (filled circles). Untransformed data. See regression equations in Table 6.7b.

Table 6.7 Regression equations and statistics for the accumulation of copper with increasing concentrations over a fixed time (6 days) in *Littorina saxatilis* from five sites in the Isle of Man.

Site	n	Equation	r^2 (%)	F-value	p<
(a) All concentrations included		a b			
Castletown	15	logy = 2.03 + 0.663 logx	37.9	7.94	0.05
Derbyhaven	15	logy = 1.64 + 1.330 logx	70.3	30.74	0.001
Laxey	15	logy = 1.87 + 0.844 logx	48.4	12.18	0.01
Peel	15	logy = 1.87 + 0.975 logx	68.6	28.64	0.001
Ramsey	15	logy = 2.03 + 0.693 logx	55.8	16.44	0.001
(b) values for 1.0 mgl^{-1} Cu excluded					
Castletown	12	y = 98.6 + 182 x	79.1	37.92	0.001
Derbyhaven	12	y = 35.7 + 147 x	98.1	520.44	0.001
Laxey	12	y = 67.6 + 143 x	79.6	38.91	0.001
Peel	12	y = 69.2 + 130 x	78.2	35.88	0.001
Ramsey	12	y = 101.0 + 140 x	71.8	25.46	0.001

Table 6.8 Analysis of Covariance (ANCOVA) to test for significant differences in copper accumulation in *Littorina saxatilis* from five sites in the Isle of Man exposed to a range of copper concentrations for six days.

(a) ANCOVA for regressions including values for 1.0 mgl^{-1} Cu for log(x+1) transformed data

Source of Variation	df	SeqSS	AdjSS	AdjMS	F	P-value
Concentration	1	0.1934	0.1934	0.1934	10.65	<0.01
Site	4	1.13261	0.74117	0.18529	10.20	<0.001
SitexConcentration	4	0.02587	0.02587	0.00647	0.36	0.839
Error	65	1.18061	1.18061	0.01816		
Total	74	2.5325				

(a) ANCOVA for regressions with values for 1.0 mgl^{-1} Cu excluded. Untransformed data

Source of Variation	df	SeqSS	AdjSS	AdjMS	F	P-value
Concentration	1	52015	52015	52015	205.31	<0.0001
Site	4	38131	20677	5169	20.4	<0.001
SitexConcentration	4	747	747	187	0.74	0.571
Error	50	12667	12667	253		
Total	59	103561				

N.B. A significant interaction between concentration and site indicates a significant difference between regression slopes.

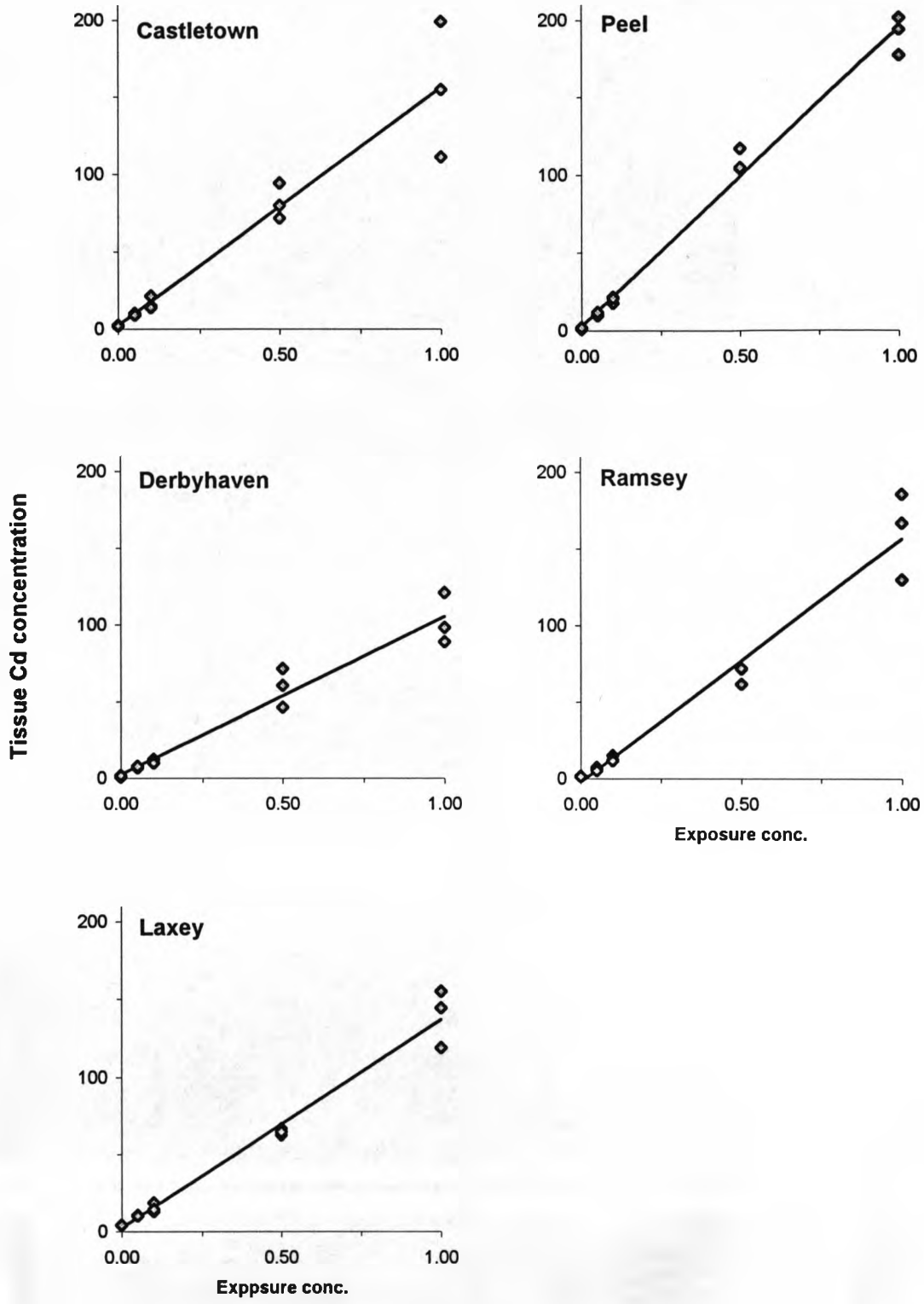

Fig 6.7 Regressions of tissue cadmium burdens ($\mu g g^{-1}$ dry weight) with increasing exposure concentration (mgl^{-1} Cd) in *Littorina saxatilis* from five sites in the Isle of Man. Animals were exposed to different cadmium concentrations for six days. See Table 6.9 for regression equations.

Table 6.9 Regression equations and statistics for the accumulation of cadmium from solution in *Littorina saxatilis* from five sites in the Isle of Man.

Site	n	Equation	r^2 (%)	F-value	p<
		a b			
Castletown	15	y = 2.40 + 154 x	92.4	158.5	0.0001
Derbyhaven	15	y = 2.59 + 103x	95.9	300.3	0.0001
Laxey	15	y = 2.50 + 134x	98.0	628.8	0.0001
Peel	15	y = 2.86 + 193x	99.0	1340.3	0.0001
Ramsey	15	y = -2.28 + 159x	96.4	351.1	0.0001

Table 6.10 Analysis of Covariance (ANCOVA) to test for significant differences in cadmium accumulation in *Littorina saxatilis* from five sites in the Isle of Man exposed to a range of cadmium concentrations for six days.

Source of Variation	df	SeqSS	AdjSS	AdjMS	F	P-value
Site	1	7102	163	41	0.30	0.876
Concentration	4	237856	237856	237856	1762.92	<0.001
SitexConcentration	4	9463	9463	2366	17.53	<0.001
Error	65	8770	8770	135		
Total	74	263192				

See note on Table 6.8

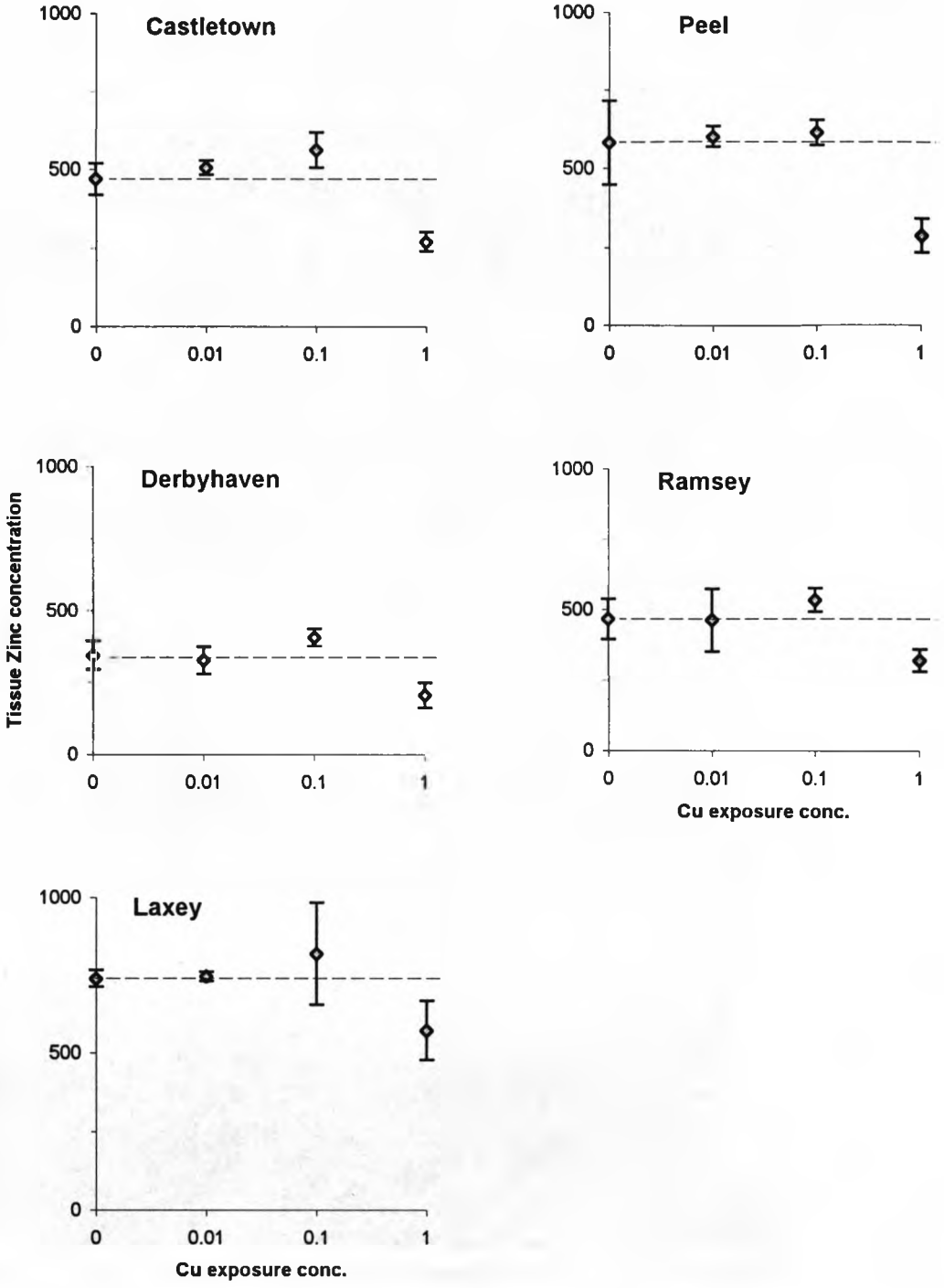

Fig 6.8 Concentrations of Zn in *Littorina saxatilis* from five sites exposed to 2mgl^{-1} added Zn and combined solutions of 2mgl^{-1} Zn and different concentrations of added Cu. Tissue Zn concentrations are in μgg^{-1} dry weight; values are mean ± s.d., n=3. Cu exposure concentrations in mgl^{-1}.
Dotted lines represent tissue Zn conc. in Zn only solution.

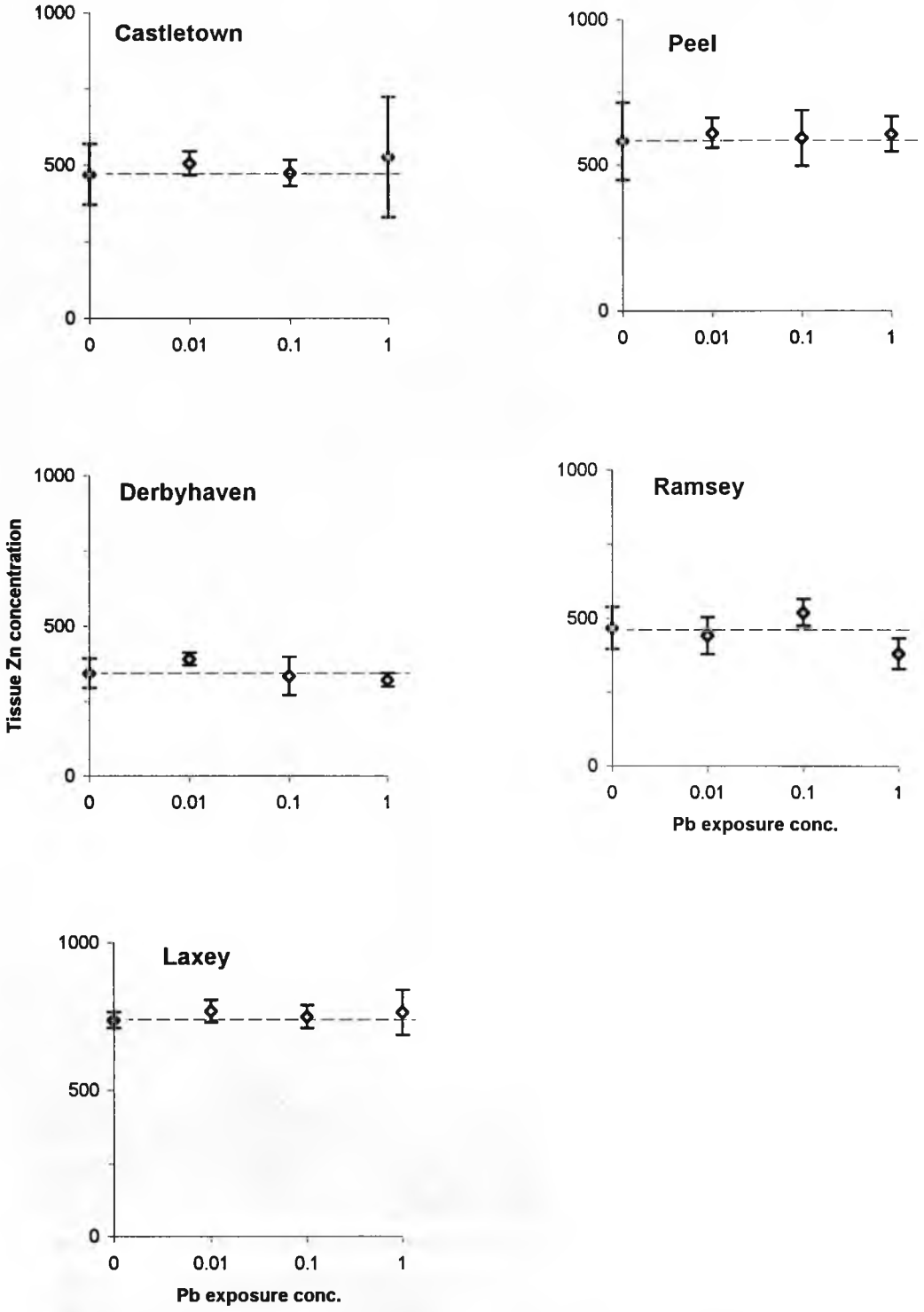

Fig 6.9 Concentrations of Zn in *Littorina saxatilis* from five sites exposed to 2mgl^{-1} added Zn and combined solutions of 2mgl^{-1} Zn and different concentrations of added Pb. Tissue Zn concentrations are in μgg^{-1} dry weight; values are mean ± s.d., n=3. Pb exposure concentrations in mgl^{-1}.
Dotted lines represent tissue Zn conc. in Zn only solution.

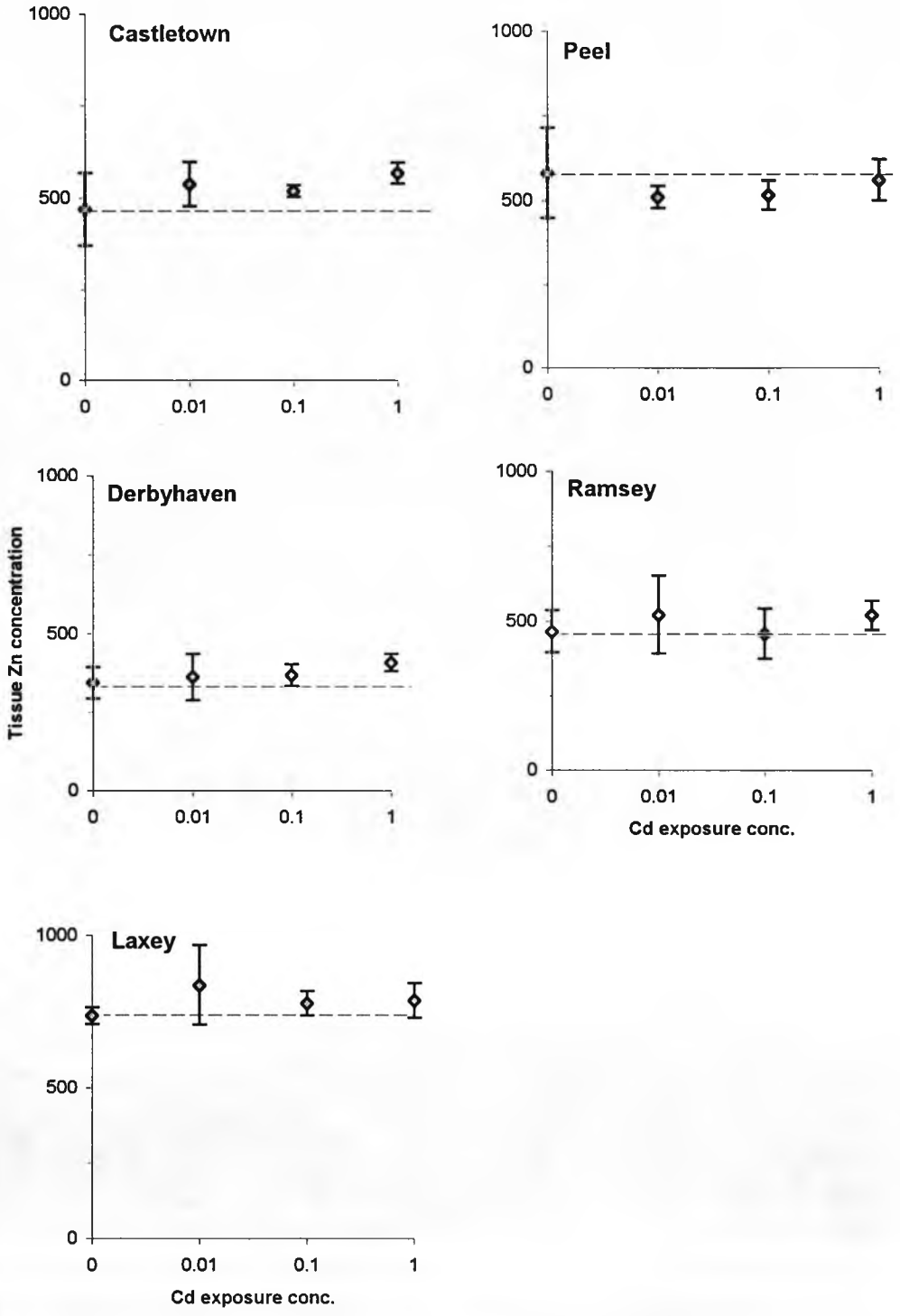

Fig 6.10 Concentrations of Zn in *Littorina saxatilis* from five sites exposed to $2mgl^{-1}$ added Zn and combined solutions of $2mgl^{-1}$ Zn and different concentrations of added Cd. Tissue Zn concentrations are in μgg^{-1} dry weight; values are mean $\pm$ s.d., n=3.
Cd exposure concentrations in mgl^{-1}.
Dotted lines represent tissue Zn concentration in Zn only solution.

At 1mg l^{-1} Cu, Zn accumulation dropped to values much lower than values in other combinations and for Zn only. This pattern of Zn accumulation was consistent for animals from all sites.

Pb did not appear to have much effect on Zn accumulation and slight differences were obtained between sites (Fig. 6.9). There was a general tendency for Zn accumulation to be higher in Pb/Zn combined solutions than for accumulation from Zn only solutions. No appreciable increase in Zn accumulation occurred with an increase in the concentration of Pb and in animals from Derbyhaven and Ramsey no effect was apparent. Zn accumulation in Cd/Zn solutions were also generally enhanced above values in solutions of Zn only (Fig. 6.10); accumulation in the animals from Peel being the only exception.

6.3.3 Effect of Zn on Cu, Pb and Cd accumulation

Reverse experiments were not conducted to evaluate the effects of increasing concentrations of Zn on Cu, Pb and Cd accumulation in *L. saxatilis*. However, the respective metal burdens were measured in the experiment where different concentrations of the metals were combined with a constant Zn concentration. A comparison of metal values in that experiment with values obtained for the accumulation of the respective metals in single exposures could give an insight into the effects of Zn on the accumulation of these metals.

Figs. 6.11 to 6.13 show that in combinations with a constant concentration of Zn ($2mgl^{-1}$ Zn), the accumulation of Cu, Pb and Cd generally increased with an increase in

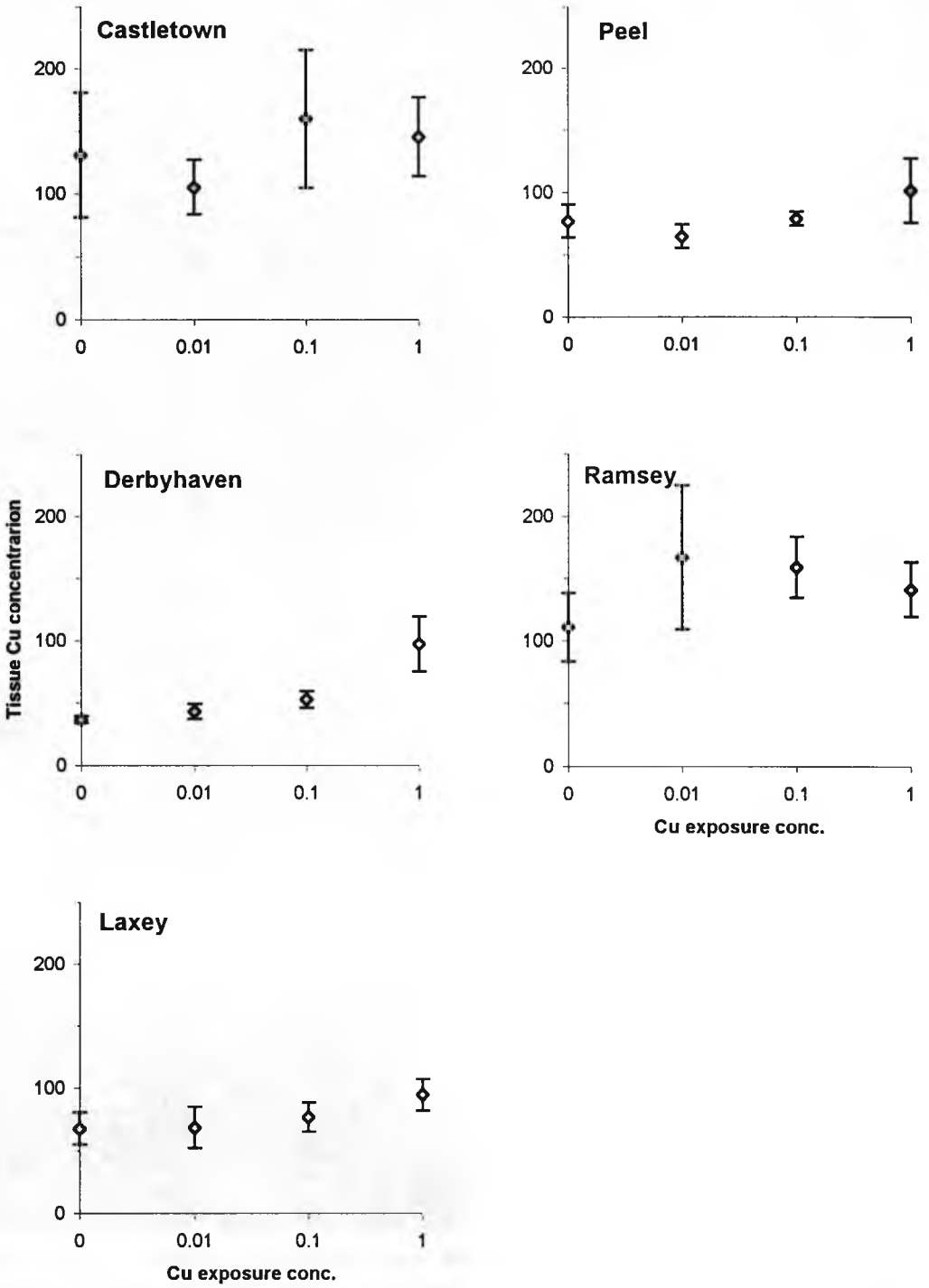

Fig 6.11 Concentrations of Cu in *Littorina saxatilis* from five sites exposed to combined solutions of a constant Zn concentration ($2mgl^{-1}$ Zn) and different concentrations of added Cu. Tissue Cu concentrations are in μgg^{-1} dry weight; values are mean $\pm$ s.d., n=3. Cu exposure concentrations in mgl^{-1} .

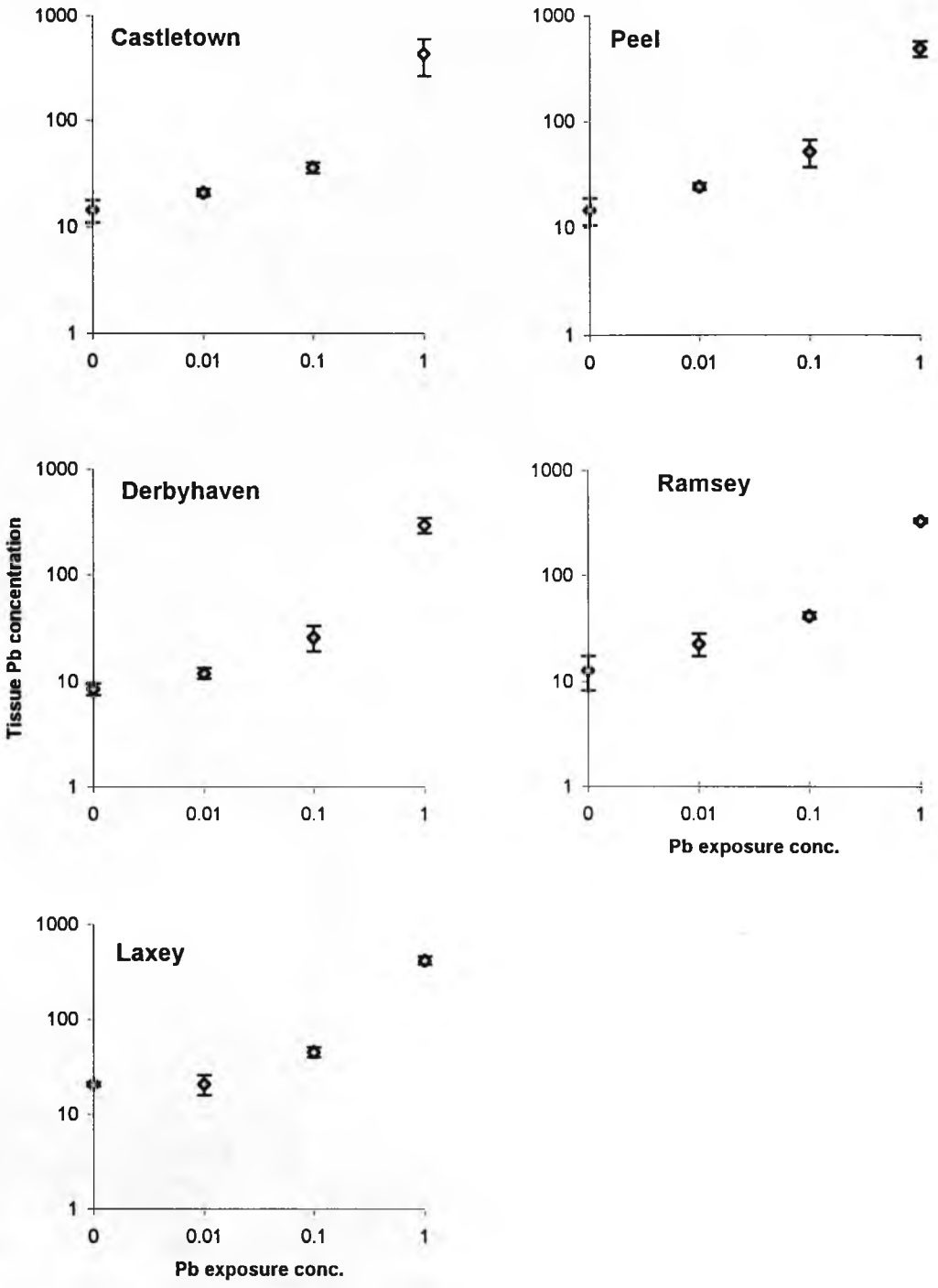

Fig 6.12 Concentrations of Pb in *Littorina saxatilis* from five sites exposed to combined solutions of a constant Zn concentration ($2mgl^{-1}$ Zn) and different concentrations of added Pb. Tissue Pb concentrations are in μgg^{-1} dry weight; values are mean $\pm$ s.d.(n=3) plotted on a logarithmic scale. Pb exposure concentrations in mgl^{-1}.

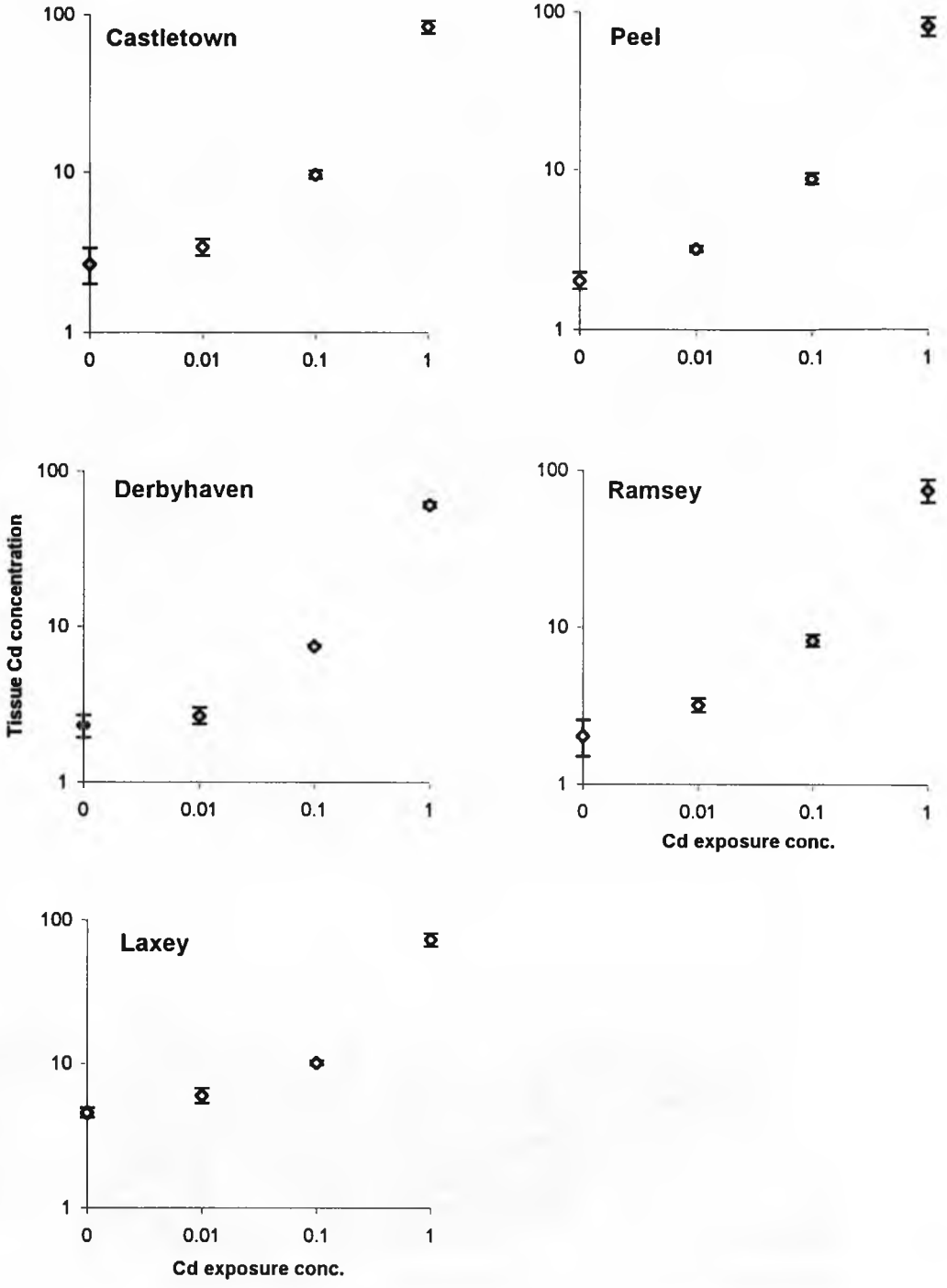

Fig 6.13 Concentrations of Cd in *Littorina saxatilis* from five sites exposed to combined solutions of a constant Zn concentration ($2mgl^{-1}$ Zn) and different concentrations of added Cd. Tissue Cd concentrations are in $\mu g g^{-1}$ dry weight; values are mean $\pm$ s.d.(n=3) plotted on a logarithmic scale. Cd exposure concentrations in mgl^{-1}.

the respective metal concentrations. In the case of Cu, some differences in response occurred between different populations (Fig. 6.11). Animals from Castletown and Peel showed an initial drop in Cu burdens below values for Zn only solutions (which were approximately initial values) followed by an increase, whereas for animals from Laxey and Derbyhaven such a drop was not observed. Individuals from Ramsey showed an anomalous response whereby Cu accumulation appeared to reduce with increasing concentration of Cu in solution

Comparisons of Cu, Pb and Cd accumulation alone with accumulation in combinations with $2mgl^{-1}$ Zn were made for $0.1mgl^{-1}$ and $1mgl^{-1}$ of the respective metals (Table 6.11a-c). Because initial metal levels in the animals were slightly different in the two experimental series, the difference in tissue metal burdens and initial levels (Δc) in the animals are included for comparison. Table 6.11a shows that Zn had minimal effect on Cu accumulation although animals showed an inconsistent pattern between sites. Pb accumulation (Table 6.11b and Cd accumulation (Table 6.11c) were both reduced in the presence of 2 mgl^{-1} Zn in comparison with the accumulation of the respective metals alone in solution. No inter-site differences were apparent. The percentage increase or reduction of accumulation of each metal was calculated as:

$$\partial\Delta_c(\%) = \frac{\Delta_{c1} - \Delta_{c2}}{\Delta_{c1}} \times 100$$

where $\Delta c1$= net uptake from metal only solution

$\Delta c2$= net uptake from metal/Zn solution

$\delta\Delta c$ values were calculated for the accumulation of Cu, Pb and Cd at concentrations of 0.1 and 1.0 mg l^{-1} of each metal and the values are plotted in Fig. 6.14. The erratic

Table 6.11 Comparisons of metal accumulation levels between values obtained for exposure to individual metals and values obtained in metal+ 2mgl^{-1} Zn. Left side =0.1 and right side =1.0 mgl^{-1} metal concentraion.

(a) copper

Site	Tissue Cu conc. Cu only	Cu+2mgl^{-1} Zn	Δ_{C1}	Δ_{C2}	Tissue Cu conc. Cu only	Cu+2mgl^{-1} Zn	Δ_{C1}	Δ_{C2}
Castletown	116.7± 16.2	159.9 ± 55.2	40.38	55.40	145.9 ± 30.5	145.4 ± 31.5	69.56	40.89
Derbyhaven	50.3 ± 5.5	52.6 ± 6.7	16.05	13.45	85.2 ± 2.0	97.2 ± 22.2	50.98	57.98
Laxey	81.9 ± 9.0	76.9 ± 11.5	10.06	21.82	110.4 ± 13.8	94.7 ± 12.8	38.55	39.69
Peel	80.5 ± 9.2	78.8 ± 5.7	-5.28	18.60	128.0 ± 5.9	101.3 ± 26.0	42.25	41.15
Ramsey	118.9 ± 26.0	158.7 ± 24.3	8.00	62.44	154.6 ± 11.7	141.0 ± 21.9	43.65	44.73

(b) lead

Site	Tissue Pb conc. Pb only	Pb+2mgl^{-1} Zn	Δ_{C1}	Δ_{C2}	Tissue Cu conc. Pb only	Pb+2mgl^{-1} Zn	Δ_{C1}	Δ_{C2}
Castletown	62.8 ± 6.6	35.7 + 4.1	50.47	31.96	595.3 ± 27.0	434.7 ± 170.0	582.93	430.92
Derbyhaven	37.8 ± 7.3	25.8 ± 6.8	31.20	21.94	424.7 ± 28.3	291.3 ± 47.7	418.05	287.41
Laxey	61.3 ± 5.0	44.7 ± 5.5	42.10	31.25	585.2 ± 38.5	414.3 ± 42.7	566.07	400.93
Peel	57.0 ± 5.3	51.0 ± 14.8	41.77	34.07	607.9 ± 33.3	493.0 ± 84.4	592.67	476.04
Ramsey	44.8 ± 6.1	41.6 ± 3.0	34.59	38.40	515.6 ± 28.2	330.5 ± 16.2	505.43	327.34

(c) cadmium

Site	Tissue Cd conc. Cd only	Cd+2mgl^{-1} Zn	Δ_{C1}	Δ_{C2}	Tissue Cu conc. Cd only	Cd+2mgl^{-1} Zn	Δ_{C1}	Δ_{C2}
Castletown	17.0 ± 4.0	9.70 ± 0.59	14.70	7.39	155.2 ± 44.0	84.16 ± 7.93	152.91	81.85
Derbyhaven	11.6 ± 1.5	7.48 ± 0.01	10.16	5.41	102.9 ± 16.5	60.48 ± 2.51	101.46	58.41
Laxey	15.4 ± 2.9	10.09 ± 0.36	11.11	4.99	139.3 ± 18.5	73.04 ± 7.82	135.06	67.94
Peel	20.2 ± 2.0	8.74 ± 0.67	18.52	7.22	191.5 ±12.5	82.14 ± 11.03	189.80	80.63
Ramsey	13.0 ± 1.9	8.19 ± 0.68	11.49	6.33	160.7 ± 28.4	73.98 ± 12.09	159.12	72.12

Δ_{C1}= differences in tissue metal concentration (from initial values) after exposure to respective metals only in solution.

Δ_{C2} = differences in tissue metal level (from initial values) after exposure to metal+2mgl^{-1} Zn in solution.

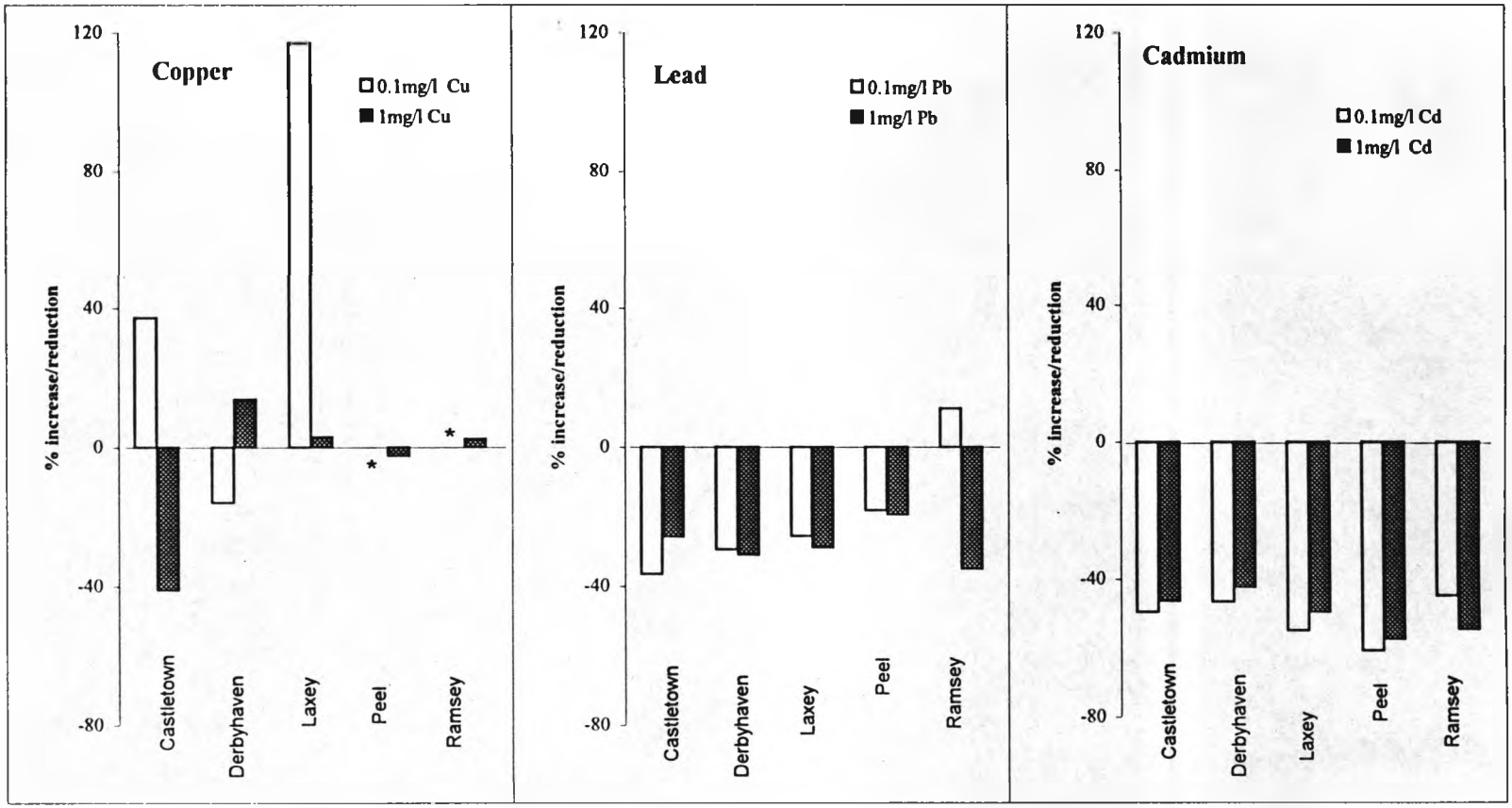

Fig 6.14 Effects of 2 mgl^{-1} zinc on the accumulation of copper, lead and cadmium in *Littorina saxatilis* at 0.1 mgl^{-1} and 1 mgl^{-1} of the respective metals. Values plotted are percentage differences in $\delta\Delta_C$ (see text). * refer to extreme values not plotted (see Table 6.9a).

effect of Zn on Cu accumulation is confirmed and percentage reductions in accumulation were shown to be higher for Cd than for Pb accumulation.

6.4 DISCUSSION

The rate of net uptake of stable Zn from solution was slower for the Zn tolerant *Littorina saxatilis* from Laxey and this was indicated by significantly lower regression slopes (log-tissue concentration against log-time) for exposures to 2.5 mgl^{-1} and 5.0 $mg\ l^{-1}$ added Zn. Thus tolerant animals accumulated less Zn than non-tolerant individuals from other sites around the Isle of Man. This is in agreement with previous studies of relative Zn accumulation in tolerant animals from Dulas Bay (Robinson, 1985; Webb, 1990) and Laxey (Webb, 1990) using radio-labelled Zn (^{65}Zn). Cu tolerance in the Dulas Bay winkles was also associated with reduced uptake of that metal (Webb, 1990). Other species of aquatic invertebrates have been reported to exhibit reduced permeability as a mechanism of metal tolerance (see Abdullah & Ireland, 1986; Credland, 1988; Bryan & Gibbs, 1983; Naylor, 1987).

In contrast to Zn tolerance, tolerance to Pb was found to be associated with a higher rate of accumulation of Pb in animals from Laxey in comparison to those from control sites. It was suggested in chapter four that tolerance to Pb may be independent or linked to Zn tolerance. Bryan (1976b) reported that in *N. diversicolor* from Restronguet Creek where tolerance to silver depends on tolerance to Cu and probably Pb, silver uptake was higher in the tolerant than non-tolerant worms. This was suggested to be an indication that the excess metal binding capacity in the tolerant worms was being titrated with silver. While it may be true that Pb is being sequestered on an excess metal binding capacity in tolerant *Littorina* from Laxey, it cannot be conclusively stated that such excess capacity is entirely the result of Zn burdens. While initial silver levels in Restronguet Creek worms were lower than or similar to levels in

the worms from control sites, the same is not true of Pb burdens in *L. saxatilis* from Laxey as they had the highest initial Pb levels.

In experiments with *Nereis diversicolor*, uptake of ^{64}Cu was found to be higher in tolerant worms (from Restronguet Creek) than non-tolerant (from Avon estuary) and this was thought to reflect the greater capacity of tolerant animals for binding and detoxifying Cu in the epidermal cells (Bryan, 1976b). However, animals from the Tamar estuary which were non-tolerant but contained a high concentration of Cu, also absorbed Cu rapidly from solution. When the experiments were repeated with laboratory grown animals in which the level of Cu had been reduced by growth dilution, uptake of Cu from low external concentrations was lower in the Restronguet Creek animals than those from the Avon estuary. This led Bryan (1976b) to suggest that the persistence of tolerance of the Restronguet Creek animals could have its basis in a low permeability to Cu which is normally masked by the greater tendency for high-Cu animals to absorb and bind Cu in the epidermal cells. It is not possible to suggest on the basis of the evidence from this work that such an underlying lower permeability to Pb might exist in the tolerant populations.

Brown (1978) observed that the nature of interactions between metals may give an indication of the mechanism of co-tolerance between metals. In the population of the freshwater isopod, *Asellus meridianus* from River Hayle, tolerance to Pb is conferred as co-tolerance to Cu (Brown, 1978). She found that Cu-tolerant isopods from the Hayle "detoxify" Pb by storing the metal in "cuprosomes" at the expense of Cu. Hence, in mixed solutions of an increasing concentration of Pb and a fixed concentration of Cu, the animals progressively took up more Pb while the

accumulation of Cu dropped. This contrasted with the response of animals from the Gannel, a site with high Pb levels which also exhibited tolerance to Pb. The Gannel animals were generally less permeable to Pb under those conditions than the Hayle animals and Pb accumulation did not appear to antagonise Cu accumulation. In my experiments with mixtures of a constant Zn concentration and an increasing Pb concentration, Pb did not appear to affect the accumulation of Zn and there was no appreciable difference in Zn accumulation by the tolerant animals from Laxey. Zn was observed to have an antagonistic effect on Pb accumulation but again the snails from Laxey did not appear to show any deviation from the general trend. It is therefore unlikely that tolerance to Zn necessarily confers co-tolerance to Pb which may have developed independently. This is supported by the fact that tissue Pb burdens were highest in the Laxey population although this was only significant in comparison with two populations (see Chapter 3).

The net uptake of Cu over time in *L. saxatilis* was erratic and no inter-site differences were apparent. The absence of inter-site differences was expected since no clear-cut differences in susceptibility to Cu occurred between the different populations. The accumulation of the metal with increasing concentration showed that above a certain concentration, the animals were able to detect the presence of Cu and "actively" attempt to reduce its uptake. Hence, the net uptake at 1mg l^{-1} Cu was less than that at 0.5 mg l^{-1} Cu. Whether such a reduction in accumulation is the result of physiological mechanisms is unknown but it is likely that behavioural responses (retraction and shell closure) are important. The interaction between Zn and Cu in combined solutions tend to support the suggestion that behavioural responses to reduce Cu uptake may occur in

L. saxatilis. In combined solutions of 2mg l^{-1} Zn and increasing concentrations of Cu (0.001 to 1.0 mg l^{-1} Cu), Zn did not generally appear to affect the accumulation of Cu although the accumulation of the latter metal was erratic. The accumulation of Zn from solutions of Zn+0.1 mg l^{-1} Cu was higher than levels in solutions of Zn only. However, at 1.0mg l^{-1} Cu + Zn, the accumulation of Zn was much lower than was obtained from 2 mg l^{-1} Zn alone. Preferential binding to Cu in competition for uptake sites is not thought to be responsible for the lower Zn accumulation, since in general, Cu accumulation in that combination did not appreciate over levels of 1mg l^{-1} Cu alone. Presumably, mechanisms primarily targeted at reducing the influx of Cu also resulted in a reduced accumulation of Zn.

Mason & Simkiss (1983) identified two types of intracellular activities, the complex interactions of which may determine the distribution and concentrations of metals in *Littorina littorea*. One type involved very specific cells, such as pore cells and connective tissue calcium cells, which occur diffusely in the connective tissue and which accumulate specific metals (i.e. Cu and Mg respectively) along precise metabolic pathways protected from the influence of other interfering metals. The former cells are involved in the metabolism of the copper-containing respiratory protein haemocyanin, whilst the latter appear to be involved in regulating pH and ionic composition of the haemolymph. Secondly, other cells, such as basophil cells of the digestive gland and the nephrocytes of the kidney, occur at specific sites and apparently produce non-specific ligands capable of binding a wide variety of metals. Similar types of ligand production might exist for *L. saxatilis*. That Cu metabolism (associated with haemocyanin) primarily involves ligands in the pore cells which is a protected pathway

not parasitized by other metals may explain why Zn did not affect Cu uptake. Also, Webb (1990) suggested that Cu may be bound to a different type of ligand (from Zn, Mn and Fe) in *L. saxatilis* since excretion of these metals occurred but no Cu excretion was observed under his experimental conditions. It is very likely therefore, that the reduction of Zn accumulation in the Zn+1mg l^{-1} Cu solution was more behavioural than physiological. Additional production of non-specific ligands may also have taken place in the low Cu+Zn combination resulting in the higher Zn accumulation from 0.1mg l^{-1} Cu/Zn solutions.

The accumulation of Cd alone in solution showed a significant difference between sites despite the absence of enhanced tolerance to this metal by individuals from any site. However, the animals from Peel which had the highest accumulation rate were also the most susceptible to Cd toxicity (see Chapter 4). In interactions between Cd and Zn, Zn accumulation appears be higher in solutions of Zn+Cd than from Zn alone, except for accumulation in animals from Peel. As with Pb, Zn had an antagonistic effect on Cd accumulation. The reduction of accumulation was greater for Cd than it was for Pb possibly because of the higher chemical similarity between Zn and Cd.

Metals may be taken up from solution or food but most laboratory experiments of metal uptake are done with metals in solution (e.g. Nugegoda & Rainbow, 1995). Pb as nitrate is the most soluble inorganic form but it is insoluble in seawater above 4.5 mg l^{-1} and on that account Mance (1987) questions the validity of toxicity experiments where effects have been reported above such values. Bryan (1976b)

speculated that *N. diversicolor* from Restronguet Creek may be tolerant to Pb because of high environmental and tissue levels but that this was not tested experimentally because Pb was not acutely toxic at 5 mg l^{-1} beyond which it was insoluble at 50 % seawater. Although the presence of undissolved Pb was observed in my experiments, I found mortality to increase with increasing concentration of Pb above 5 mg l^{-1} (10 < 20mg l^{-1}, although probably non-significant) in *L. saxatilis*. This implies that some uptake of undissolved metal may have taken place. The uptake of insoluble metal by endocytosis have been noted in several molluscan species (e.g. Fowler *et al.*, 1975; Lowe & Moore, 1979; George & Pirie, 1980). In the bivalve *Abra alba*, endocytosis of fine particles occur not only in the digestive gland but also in the gills (Martoja *et al.*, 1988). Particles of hydrous ferric oxide has been demonstrated to be taken up at a significant rate through the gills of *Mytilus edulis* by pinocytosis and transferred to other tissues via circulating amoebocytes (George *et al.*, 1976). This process appears to be energy dependent (Bryan, 1979) and the uptake of Pb has also been observed (Coombs & George, 1978). It is suggested that processes other than passive diffusion are also involved in the uptake of Pb by *L. saxatilis* in the experimental concentrations used. The metal accumulation experiments showed that Pb accumulation was monotonic with increasing concentration of added Pb. Furthermore, it appears that Pb was accumulated faster than other metals in my experiments. Zn, Cu and Cd were soluble in seawater at the concentrations used for the tolerance and accumulation experiments.

In gastropod molluscs, food is an important source of metals (Ireland, 1973; Young, 1975, 1977). In *Littorina obtusata* Zn uptake from food was shown to be up to 30 %

(Young, 1977); and the uptake of arsenic in *Littorina obtusata* (Klumpp, 1980) and Pb uptake in the abalone, *Heliotis* sp. (Stewart & Schulz-Baldes, 1976) was mainly from the food. It is not known how the relative importance of the uptake routes would affect the accumulation of the metals studied and their toxicity.

In summary, tolerance to Zn in *L. saxatilis* from Laxey was mainly through lower accumulation of the metal. In contrast, tolerance to Pb in the same population is associated with increased accumulation, and storage in inert form is thought to be the mechanism involved. The accumulation experiments also suggest that tolerance to the two metals may not be linked. In combined metal exposures, Zn was antagonistic to Pb and Cd but not Cu but the relative magnitude of antagonistic interactions between individuals from different sites appear to be unrelated to metal tolerance. Physiological and/or behavioural responses aimed at reducing Cu accumulation also resulted in a reduced accumulation of Zn.

CHAPTER SEVEN

FIELD STUDIES OF THE REPRODUCTIVE EFFECTS OF HEAVY METALS IN *LITTORINA SAXATILIS*

7.1 INTRODUCTION

Sub-lethal effects of pollutants such as depression of growth (e.g. Kaviraj & Konar, 1983, Shella *et al.*, 1995) and reproduction (e.g. Vranken & Heip, 1986; Verriopoulous & Hardouvelis, 1988) occur at concentrations where toxic effects may not be apparent. The embryonic and larval stages of aquatic animals are generally the most sensitive stages of the life cycle to heavy metals and other toxicants (Leland & Kuwabara, 1985). Hunt & Anderson (1989) established that at concentrations above 19 $\mu g\ l^{-1}$ Zn, larval metamorphosis in the abalone *Haliotis rufescens* was impaired. Oshida & Word (1982) reported that the number of offspring produced by the polychaete *Neanthes aranaceodentata* was reduced by exposure to 39 $\mu g\ l^{-1}$ of dissolved Cr (VI). The most conclusive evidence of reproductive effects in the field as a result of metals is found in the effects of the organic form of tin, tributyltin (TBT). The dogwhelk, *Nucella lapillus* exhibits "imposex", the imposition of male sexual characteristics on females, as a result of exposure to TBT. The decline of populations of *Nucella* along much of the Channel coast of Southern England was the result of TBT and virtually all populations in England showed visible effects of TBT pollution (Bryan, *et al.*, 1986, 1987, see Bryan & Langston, 1992 for reviews). Proud (1994) similarly found the development of "imposex" in Isle of Man populations of *Nucella*. Gibbs *et al.* (1991) observed that concentrations below 1 ng Sn l^{-1} TBT caused "imposex" and reproductive failure occurred at 5ng Sn l^{-1}.

It has been suggested that *Littorina saxatilis* could be a useful organism for monitoring the reproductive effects of contaminants because embryos are found in the brood pouches all year round (see Berry, 1961; Dixon & Pollard, 1985). Embryonic

development is internal and takes place in the brood pouch of the female so local pollution events would affect all stages of embryonic development. Hence, the number and size of juveniles may be affected. Most previous studies on the inter-site differences in numbers of embryo and the size of juveniles at birth have concentrated on the effects of natural differences such as shore height (e.g. Berry, 1956, 1961) or, the degree of exposure or shelter of the shores (e.g. Faller-Fritsch, 1977, Roberts and Hughes, 1980; Janson, 1985).

All stages of development from uncleaved egg to pre-emergent fully formed juveniles can be found in the brood pouch of females at the same time. However, in addition to normal stages of development, the brood pouch may contain a proportion of abnormally developed embryos. Some previous studies of the proportions of embryonic abnormality in *L. saxatilis* have shown that they may be natural occurrences unrelated to stress (e.g. Clyne & Duffus, 1979, Janson, 1985). Others have suggested that natural environmental stress (e.g. Thorson, 1946, Sokolova, 1995) or anthropogenic contamination (e.g. Dixon, 1983; Dixon & Pollard, 1985; Dixon *et al.*, 1985) can increase the frequency of abnormalities.

Despite being contaminated with heavy metals, the Laxey Estuary supports a large population of *Littorina saxatilis* which have been shown to possess enhanced tolerance to acute levels of Zn and Pb (see Chapter 4). The tolerance to acute levels of Zn was derived from prior exposure to sublethal levels. It is possible for such concentrations to exact reproductive effects. In this chapter, I present the results of inter-site differences in numbers of embryo in the brood pouches of adult females and the size of young at birth in *L. saxatilis* from sites around the Isle of Man, and show

how these may have been affected by metal contamination. Also, inter-site differences in the proportions of abnormal embryo were assessed.

7.2 MATERIALS AND METHODS

7.2.1 Fecundity

Littorina saxatilis of reproductive size (>6mm shell height) were collected from Castletown, Derbyhaven, Laxey, Peel and Ramsey from 8-10 May 1995, relaxed in 7.5 % magnesium chloride and fixed in 10 % formaldehyde-seawater solution (Reid, 1993). Animals were then preserved in 70 % ethanol until dissected. The shell height of each individual was measured, accurate to 0.1mm using vernier calipers, before cracking on a bench vice and the tissue separated from the shell. The sex was then determined, the presence of a brood pouch and penis identifying female and male respectively. The brood pouch of each female was removed into a gridded Petri dish and teased open. The contents were examined under a stereomicroscope and embryos were allocated to one of three developmental categories and counted. Five fairly distinct but arbitrary categories may be found in the brood pouch of *L. saxatilis* (Thorson, 1946; Berry, 1956; Clyne & Duffus, 1979; see Fig. 1). These are:

(i) eggs from uncleaved ova to trochophore-like ball of cells

(ii) unshelled veliger-like embryos

(iii) shelled veliger-like embryos from simple cone shaped caps on the visceral mass to well formed shells with no more than the first shell whorl complete

(iv) shelled veliger-like stages with more than the first whorl, and some reduction in the size of the velum

(v) well formed young, which had escaped from the egg capsules, with up to two complete whorls of shell and no velum.

In my enumeration, brood pouch contents were grouped into three categories as follows:

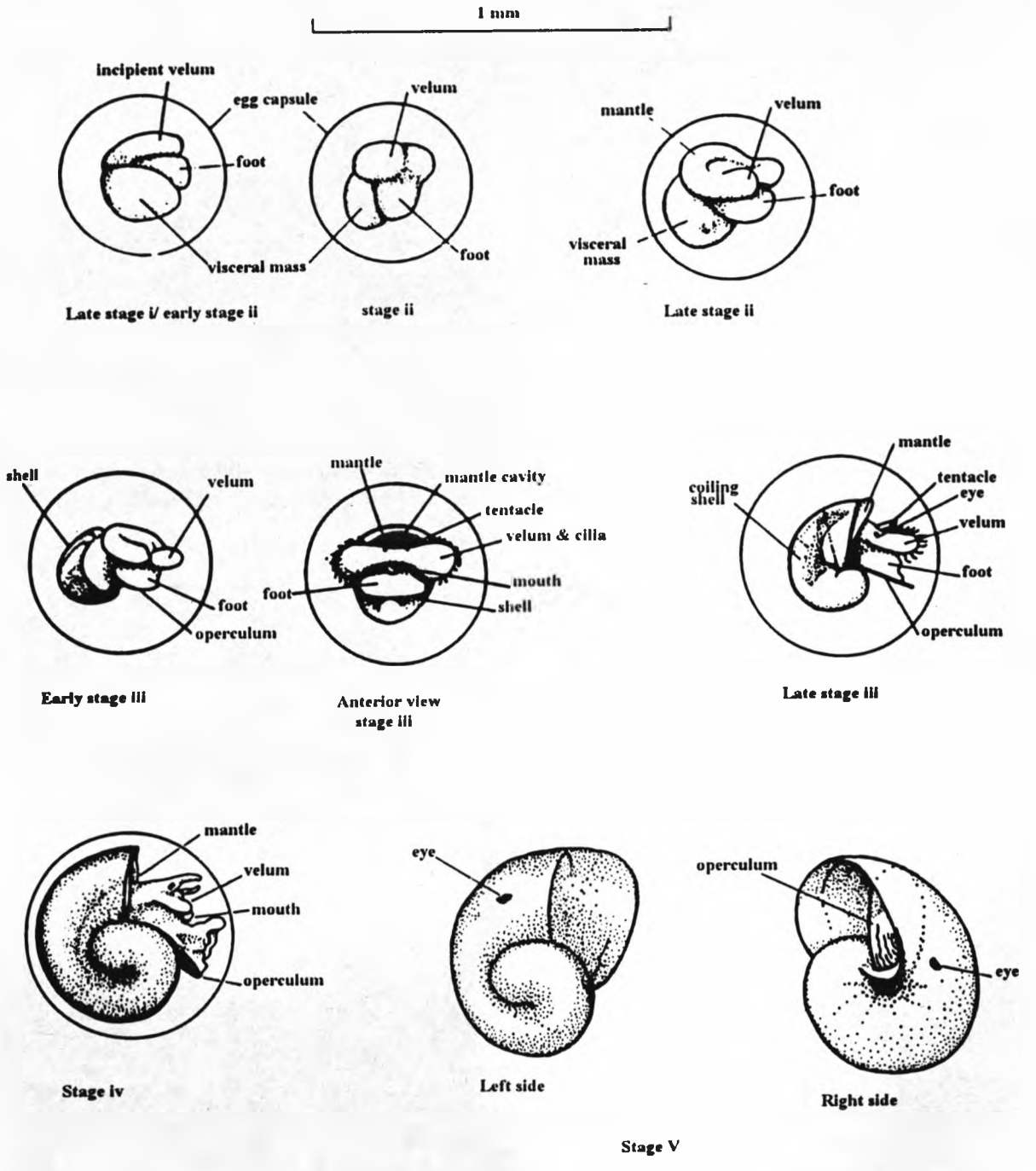

Fig 7.1 Different stages of embryonic development found in the brood pouch of *Littorina saxatilis*. See text for descriptions of the different stages. Source: Berry, 1956.

(a) unshelled embryo: egg and unshelled veliger-like stage encompassing stages (i) and (ii)

(b) shelled veliger of both the early stages and latter stages still enclosed in the egg capsule (stages iii and iv)

(c) fully formed pre-emergent young free from the egg capsule (stage v).

The tissue remaining after the brood pouch had been removed was dried to constant weight at 110 ° C in pre-weighed crucibles. The dry weight was then measured (x ± 0.001g). It should be noted that some error in dry tissue weight may have arisen from the loss of tissue during dissection.

7.2.2 Size of juveniles at birth

About fifty animals of reproductive size from each site were placed in white plastic containers after acclimatisation to 16 °C for 6 to 7 days in other tanks. The individuals transferred were thoroughly cleaned to avoid transfer of juveniles on the shells. The warm temperature was a means of inducing the free young to emerge from the brood pouch of the parents. Emergence of young from the brood pouch of the parent is entirely a function of the juvenile activity (Berry 1956, 1961). Juveniles that emerged were removed from the containers every 24 hours for five days and preserved in 70 % ethanol prior to measurement. The measurements (accurate to ± 0.001mm) were made on a stereomicroscope (Wild Heerbrugg, Finlay Microvision Ltd.) equipped with a video camera. Images were captured and calibrated in a set magnification using a NIH Image Analysis software (1.58 VDM) on a Mackintosh Computer.

7.2.3 Embryo abnormality

Embryo abnormalities were determined in samples collected on 1 July 1996. After collection, animals were stored at ambient temperature in the laboratory for a few days before dissection. The shell height of each animal was measured as above before cracking on a bench vice. Sexes were determined and the brood pouch content of females were placed in a Petri dish and examined under a stereomicroscope. Total brood pouch contents were counted and each embryo was examined for abnormality. The types of abnormalities that may be found in brood pouch of *L. saxatilis* have been described by Thorson (1946), Clyne & Duffus (1979), Dixon & Pollard (1985). Abnormalities involving unshelled embryos (mainly characterised by the physical disruption of soft tissues, resulting in the presence of a number of separate, floating, cell masses within the egg capsule; double embryos) and shelled embryos (multiple embryos mostly twins, abnormal shells mostly straight shells) were counted separately.

Data Analysis

Inter-site comparisons in brood pouch contents have been made by counting the number of embryos in a specific size range for all sites (e.g. Faller-Fritsch, 1977). Janson (1985) examined individuals in the range of sizes of reproductively mature adults in each population; and compared size and weight specific differences by plotting the regressions of $\log_e$ number of embryo against shell length or shell weight. I have adopted the latter approach for this study. Regressions of $\log_e$ total brood pouch content were plotted against shell height and tissue dry weight for animals from each site. The slope and intercept of the regression lines were compared by Analysis of

Covariance. Differences in the median absolute number of embryos and the proportions of the different stages of embryonic development were compared using the Kruskal-Wallis One-way Analysis of Variance. Where significant differences were obtained with the Kruskal-Wallis test, the Dunn non-parametric multiple comparison test was used to make pairwise comparisons between average ranks (Zar, 1984).

Significant differences in size at birth between animals from the five populations were tested by means of One-way Analysis of Variance and Tukey HSD multiple comparisons.

The proportions of abnormal embryos in each female were calculated for each of the two categories of abnormality and for total abnormality. Frequency distributions were plotted for the total abnormality in each site. Significant inter-site differences in the proportions of unshelled, shelled and total abnormality were tested using Kruskal-Wallis test and Dunn test where applicable.

7.3 RESULTS

7.3.1 Shell height measurements and fecundity counts

Size-frequency histograms showing the distribution shell heights in samples collected for the fecundity counts in May 1995 and those collected for embryo abnormality examination in July 1996 are presented in Figures 7.2 and 7.3 respectively. The size distributions are different for the five sites and animals from Castletown were the largest while those from Peel were the smallest. Larger samples were obtained in July 1996 in comparison with those obtained in May 1995 although the relative sizes of animals between sites was generally similar.

The absolute numbers of embryos in the brood pouch animals from the five sites were variable with total counts ranging overall from 24 to 1117 (see Table 7.1). Animals from Laxey, the site at which *L. saxatilis* were more exposed to heavy metal contamination had the highest mean 'total' number of embryos in both May 1995 and July 1996 samples. The mean absolute brood pouch content recorded in July 1996 was generally similar to values for May 1995 except for numbers in samples from Peel and Castletown (Fig. 7.4a). In animals from these two sites marked increases in mean "total" number of embryo were recorded, which were coincident with the relative increase in the shell height of females from which the embryo were obtained (Fig. 7.4b). Kruskal-Wallis tests on median total brood pouch content showed significant differences for both sets of samples but at different levels, $P < 0.0001$ for May 1995

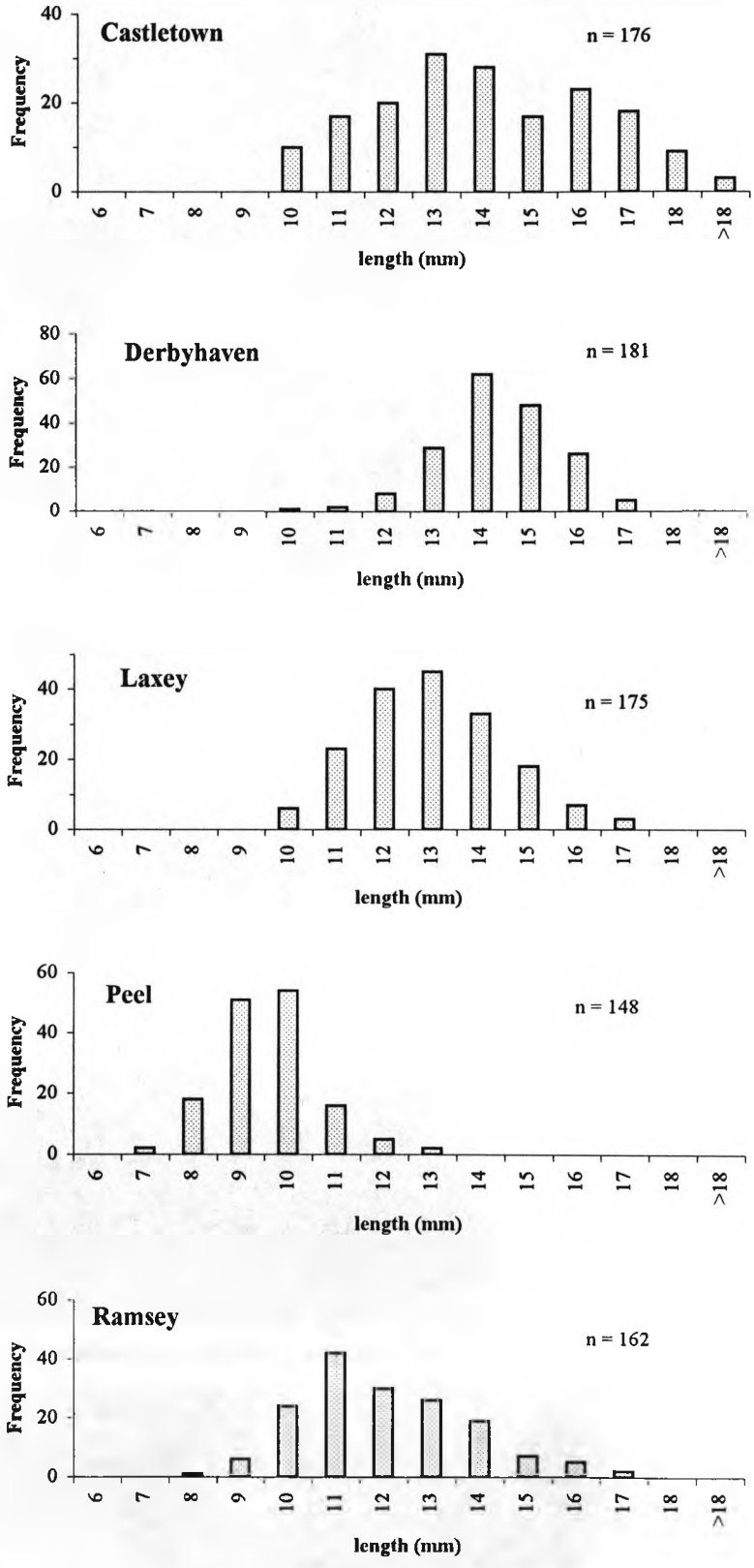

Fig 7.2 Length-frequency distributions of adult-sized *Littorina saxatilis* from five sites in the Isle of Man, collected in May 1995.

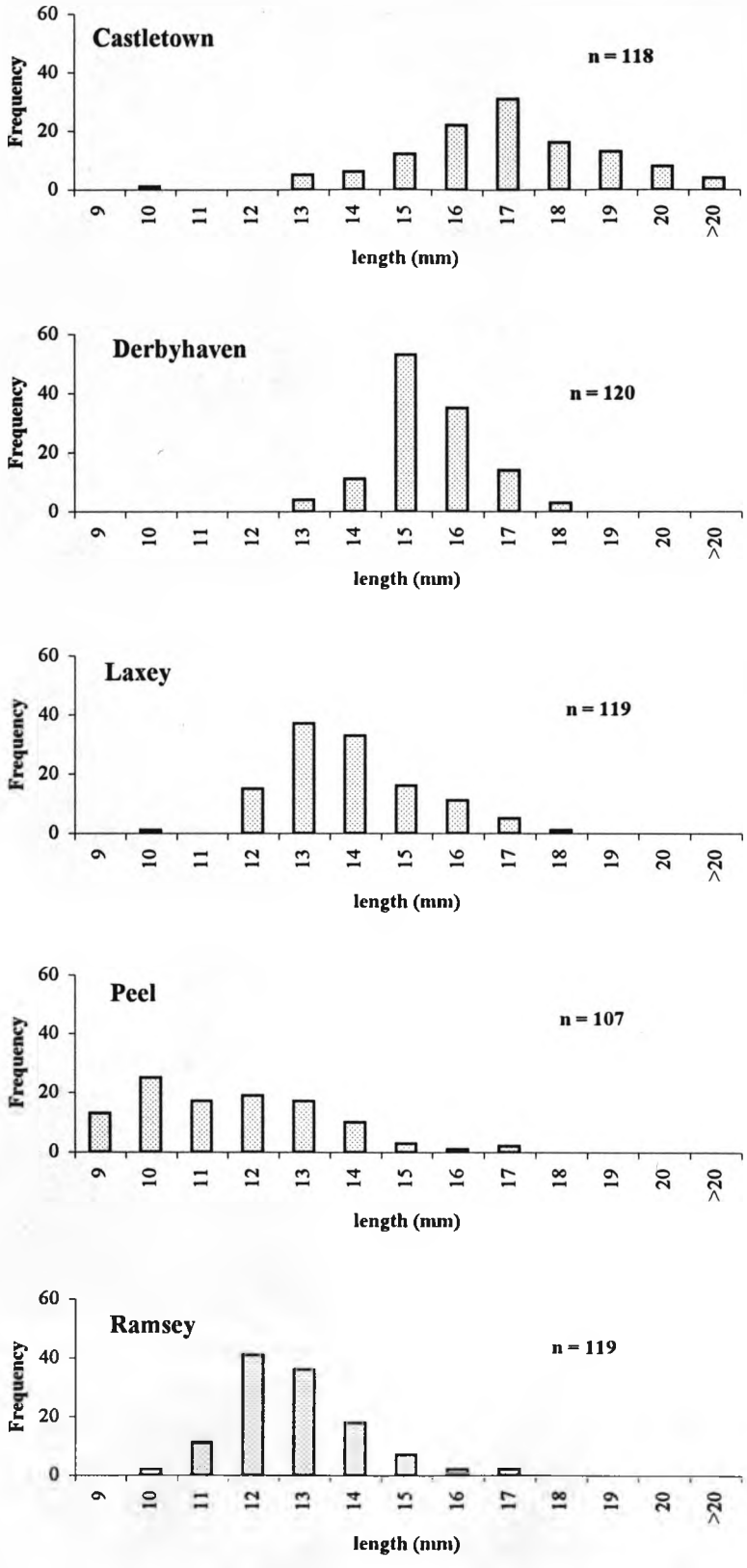

Fig 7.3 Length-frequency distributions of adult-sized *Littorina saxatilis* from five sites in the Isle of Man, collected in July 1996.

Table 7.1 Total brood pouch content of *Littorina saxatilis* from five sites in the Isle of Man in May 1995 and July 1996. Highest mean value in bold

May 1995

Site	n	minimum	maximum	mean	C.V. (%)
Castletown	45	24	652	255.22	58.31
Derbyhaven	84	54	731	277.26	49.50
Laxey	45	52	955	**435.25**	44.69
Peel	73	27	396	145.14	46.58
Ramsey	44	39	1018	316.16	58.12

July 1996

Site	n	minimum	maximum	mean	C.V. (%)
Castletown	39	46	798	359.41	45.66
Derbyhaven	63	38	690	289.71	47.31
Laxey	52	80	1117	**432.02**	59.54
Peel	46	70	835	357.26	56.01
Ramsey	50	115	580	305.56	34.30

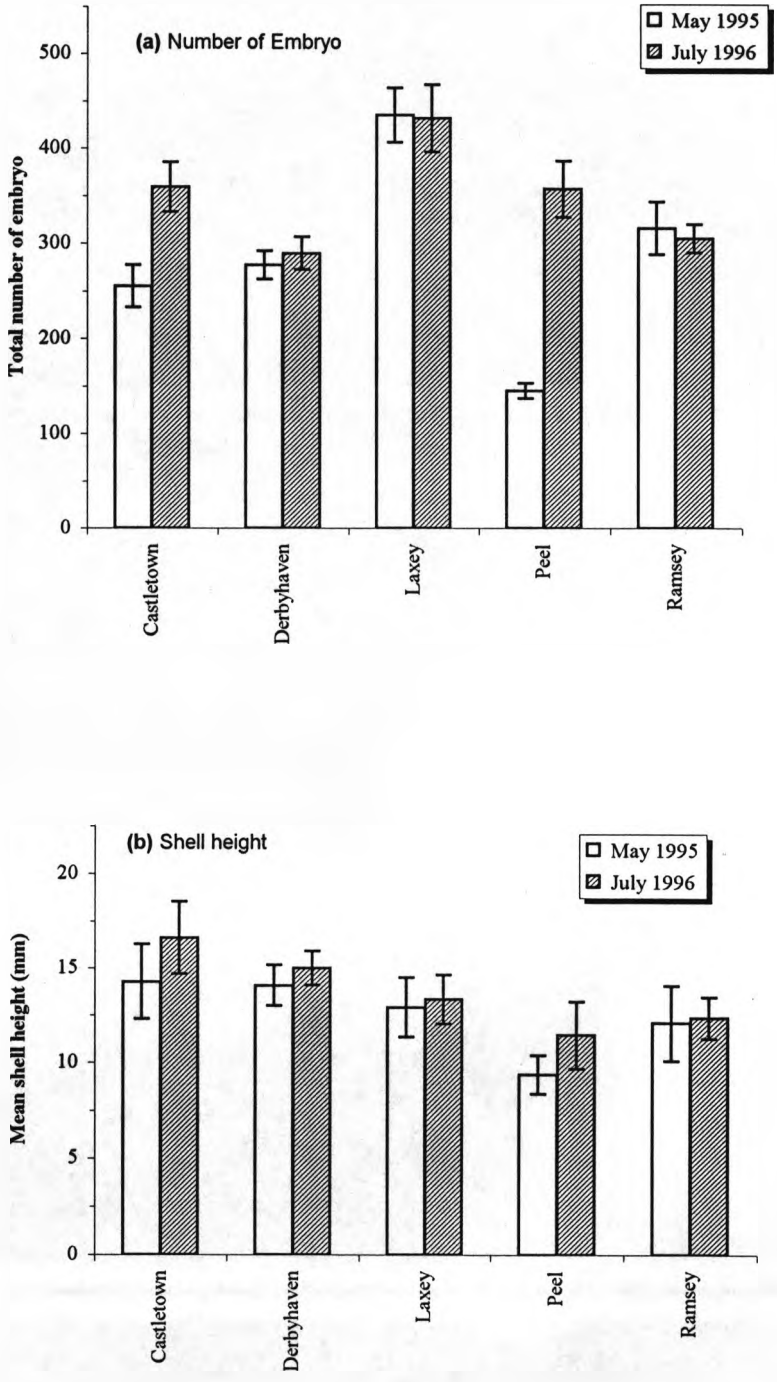

Fig 7.4 Differences in mean "total" number of embryo (± s.e.m.) in brood pouch (a), and mean (± s.d.) shell height (b), in *Littorina saxatilis* from various sites in the Isle of Man collected in May 1995 and early July 1996.

and $p < 0.05$ for July 1996. Dunn multiple comparisons on the May data showed that pair-wise significant differences in median total numbers of embryo followed the form:

Laxey > Ramsey = Derbyhaven = Castletown > Peel

(Table 7.2a). However, the Dunn test could not detect any significant differences in medians for the July data in spite of the significant differences shown by the Kruskal-Wallis test (Table 7.2b). This means that the Dunn test was less powerful and was more likely to produce a type II error (see Zar, 1984). This may have been the case since the level of significance of the Kruskal-Wallis test was low.

The numbers of the unshelled embryo, shelled veligers and pre-emergent young in the brood pouches were also variable (see Table 7.3). The mean proportions of each embryonic stage in samples from the different sites are presented in Fig. 7.5. Although Kruskal-Wallis test showed a significant difference in the proportions of unshelled embryos between sites (see Table 7.4a), there was no significant correlation between total brood pouch content and the proportion of unshelled embryos ($n=5$, $r=0.786$, $p>0.05$). The proportions of shelled embryos and pre-emergent young did not conform to the same trend as the total brood pouch content; this is shown by negative but non-significant correlations (% shelled embryo/total count: $n=5$, $r= -0.485$, $p>0.05$; % pre-emergent young/total count: $n=5$, $r= -0.748$, $p>0.05$). Some significant differences were, however, found in the proportions of shelled embryos and pre-emergent young between sites (see Table 7.4b & c).

Differences in size-specific numbers of total brood pouch contents between individuals from the five sites are presented as regressions in Fig. 7.6 for May 1995 and Fig. 7.7

Table 7.2 Kuskal-Wallis One-way Analysis of Variance (ANOVA) and Dunn test on mean ranks to test for differences in absolute brood pouch content (total numbers) in *Littorina saxatilis* from five sites in the Isle of man. Different tests for samples collected in May 1995 and July 1996. Levels of significance of Dunn test *=p<0.05, **=p<0.01, ***=p<0.001, ns=p>0.05

(a)Kruskal-Wallis for May 1995 samples

Site	n	Median	Mean rank
Castletown	45	256.0	142.2
Derbyhaven	84	262.0	157.2
Laxey	45	398.0	218.3
Peel	73	131.0	75.9
Ramsey	44	269.0	170.9
Total	219		146.0

H=89.27 (adjusted for ties), df=4, p<0.0001

Significance levels of Dunn test for May 1995

	Laxey	Ramsey	Derbyhaven	Castletown
Laxey				
Ramsey	**			
Derbyhaven	***	ns		
Castletown	***	ns	ns	
Peel	***	***	***	***

(a)Kruskal-Wallis July 1996 samples

Site	n	Median	Mean rank
Castletown	39	338.0	136.2
Derbyhaven	63	278.0	109.9
Laxey	52	365.5	146.7
Peel	46	320.0	127.8
Ramsey	50	287.0	112.7
Total	250		125.5

H=9.86, df=4 p=0.043*

Dunn test (Q values, corrected for ties) for July 1996

	Laxey	Castletown	Peel	Ramsey
Laxey				
Castletown	0.685			
Peel	1.291	0.534		
Ramsey	2.374	1.521	1.022	
Derbyhaven	2.716	1.785	1.276	0.204

$Q_{(5)}$ 0.05=2.807; no significant difference for any comparison

Table 7.3 Numbers of unshelled embryo, shelled veliger-like embryo and pre-emergent young in the brood pouches of *Littorina saxatilis* from five sites in the Isle of Man.

Unshelled embryo

Site	n	minimum	maximum	mean	C.V. (%)
Castletown	45	0	186	33.09	117.46
Derbyhaven	84	1	279	93.07	79.56
Laxey	45	3	491	150.25	56.90
Peel	73	0	82	23.21	88.05
Ramsey	44	4	236	81.77	55.76

Shelled verliger-like embryo

Site	n	minimum	maximum	mean	C.V. (%)
Castletown	45	1	393	120.71	60.01
Derbyhaven	84	3	212	77.60	60.05
Laxey	45	1	283	136.30	45.08
Peel	73	1	149	58.92	49.01
Ramsey	44	0	258	93.16	63.10

Pre-emergent young

Site	n	minimum	maximum	mean	C.V. (%)
Castletown	45	0	233	101.42	67.71
Derbyhaven	84	0	428	106.60	66.58
Laxey	45	0	527	152.16	64.60
Peel	73	0	208	63.01	53.84
Ramsey	44	0	524	141.23	65.71

Table 7.4 Kuskal-Wallis non-parametric One-way Analysis of Variance (ANOVA) and Dunn test on mean ranks to test for inter-site differences in the proportions of the three stages of embryo in *Littorina saxatilis* from five sites in the Isle of Man. Levels of significance of Dunn test *=p<0.05, **=p<0.01, ***=p<0.001, ns=p>0.05

Kruskal-Wallis on proportions of unshelled embryo

Site	n	Median	Ave Rank
Castletown	45	7.81	80.6
Derbyhaven	84	35.55	176.4
Laxey	45	32.41	204.9
Peel	73	12.25	99.5
Ramsey	44	25.33	171.8
Total	219		146.0

H=86.69 (adjusted for ties) p<0.0001

Significant levels of Dunn test on mean ranks of % unshelled embryo

	Laxey	Derbyhaven	Ramsey	Peel
Derbyhaven	ns			
Ramsey	ns	ns		
Peel	***	***	***	
Castletown	***	***	***	ns

Kruskal-Wallis on proportions of shelled veliger

Site	n	Median	Ave Rank
Castletown	45	46.53	218.5
Derbyhaven	84	27.31	106.2
Laxey	45	32.43	126.7
Peel	73	39.51	186.4
Ramsey	44	29.78	100.5
Total	219		146.0

H=84.25 (adjusted for ties) p<0.0001

Significant levels of Dunn test on mean ranks of % unshelled embryo

	Castletown	Peel	Laxey	Derbyhaven
Peel	ns			
Laxey	***	***		
Derbyhaven	***	***	ns	
Ramsey	***	***	ns	ns

Kruskal-Wallis on proportions of pre-emergent young

Site	n	Median	Ave Rank
Castletown	45	40.83	142.3
Derbyhaven	84	39.42	138.6
Laxey	45	33.42	95.2
Peel	73	44.52	171.1
Ramsey	44	43.78	174.3
Total	219		146.0

H=28.62 (adjusted for ties) p<0.0001

Significant levels of Dunn test on mean ranks of % pre-emergent young

	Ramsey	Peel	Castletown	Derbyhaven
Peel	ns			
Castletown	ns	ns		
Derbyhaven	*	*	ns	
Laxey	***	***	**	**

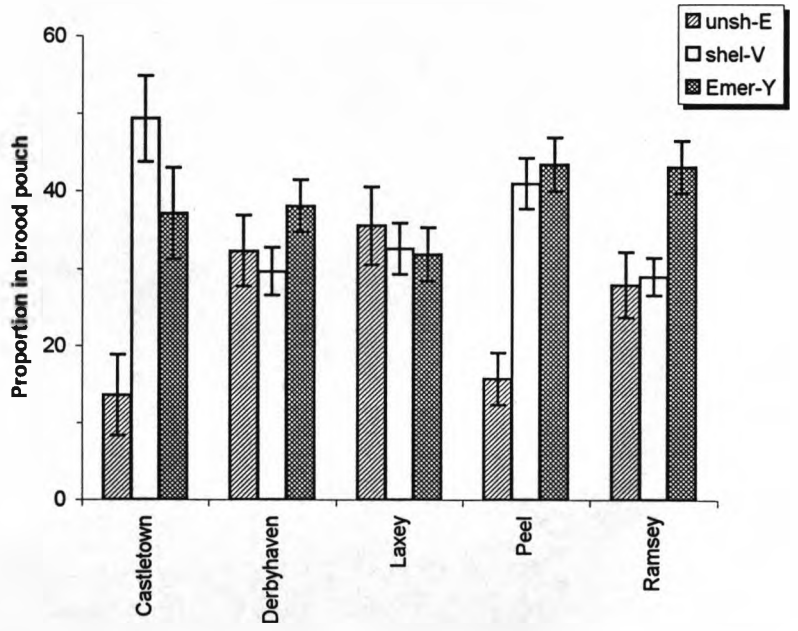

Fig. 7.5 Mean proportions of unshelled embryo, shelled veliger and pre-emergent young in the brood pouches of *Littorina saxatilis* from various sites in the Isle of Man examined in May 1995. Error bars represent s.e.m.
unsh-E = unshelled embryo
shel-V = shelled veligers
Emer-Y = pre-emergent young

for July 1996 samples. The regression equations are given in Table 7.5a and b. The slopes of the regression lines showed that animals from Laxey had the highest size-specific number of embryos in the May smples as indicated by the absolute counts. Individuals from other sites did not follow exactly the same pattern. For the July samples, animals from Peel had the highest slopes and those from Ramsey were the lowest. Analysis of Covariance did not however show any significant differences in slope or intercept for either set of samples (Table 7.6). Similarly, weight specific differences in brood pouch content between sites (see Fig. 7.8 and Table 7.7) were not significant (ANCOVA, Table 7.8). In general, despite being significant, individual regressions accounted for very low percentages of the variation in the relationship between shell height or dry tissue weight with total number of embryos as shown by the low regression coefficients (see Tables 7.5 and 7.7).

7.3.2 Size of juveniles at birth

The distributions of sizes of newly emerged juveniles (0-24 hrs old) for the five study sites are given in Fig 7.9. Mean lengths are presented in Table 7.9. Animals from Laxey (with the highest absolute pouch content) had the smallest juveniles while those from Derbyhaven were the largest. One-way Analysis of Variance showed a significant difference in the mean length at birth. Tukey multiple comparisons indicated that significant differences conformed to the following order:

Derbyhaven > Castletown > Ramsey > Peel = Laxey

(Table 7.10b).

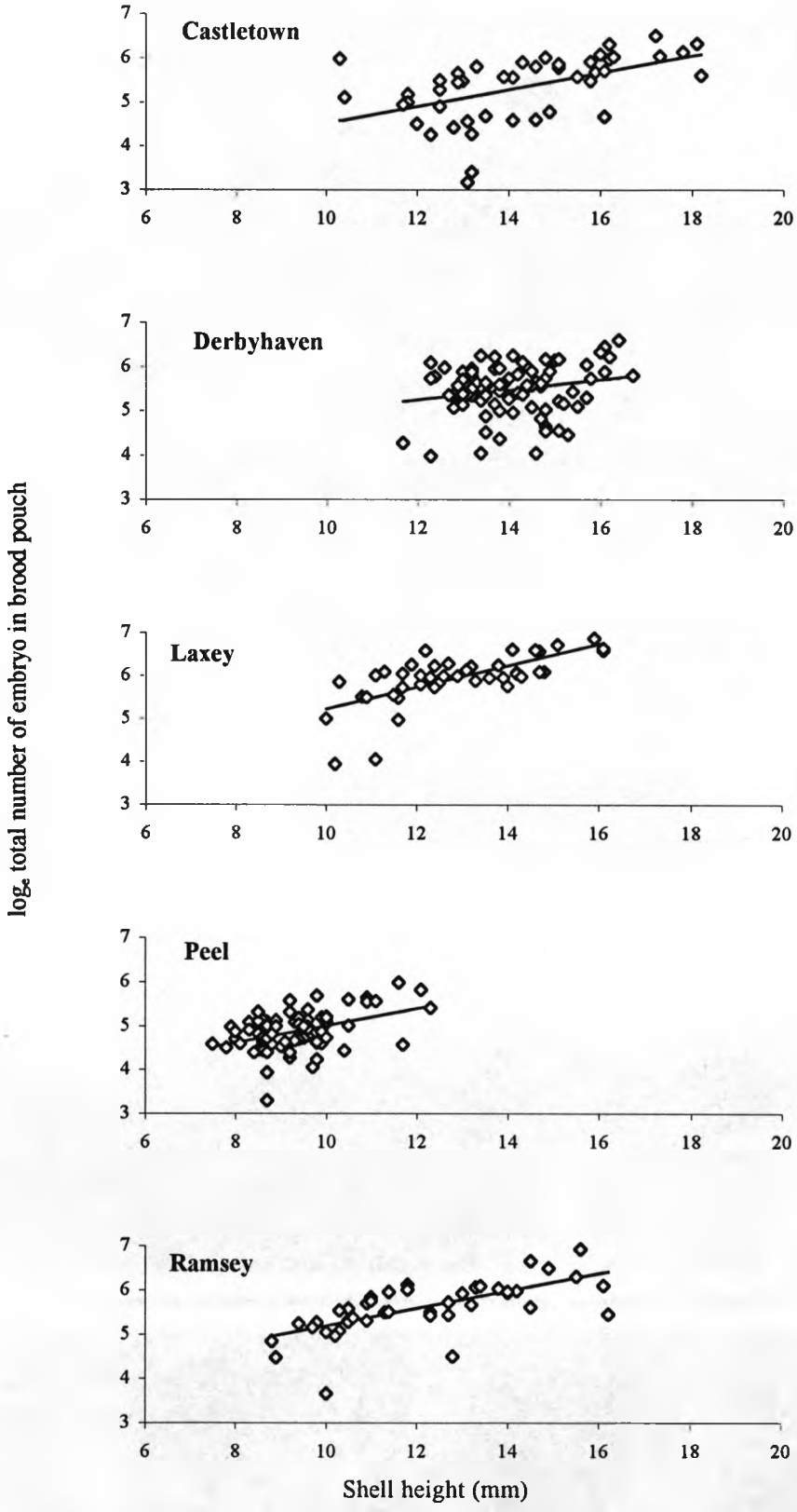

Fig. 7.6 Regressions of $\log_e$ total number of embryo in brood pouch against shell height of *Littorina saxatilis* from five sites in the Isle of Man colllected in May 1995. See regression equations in Table 7.5a.

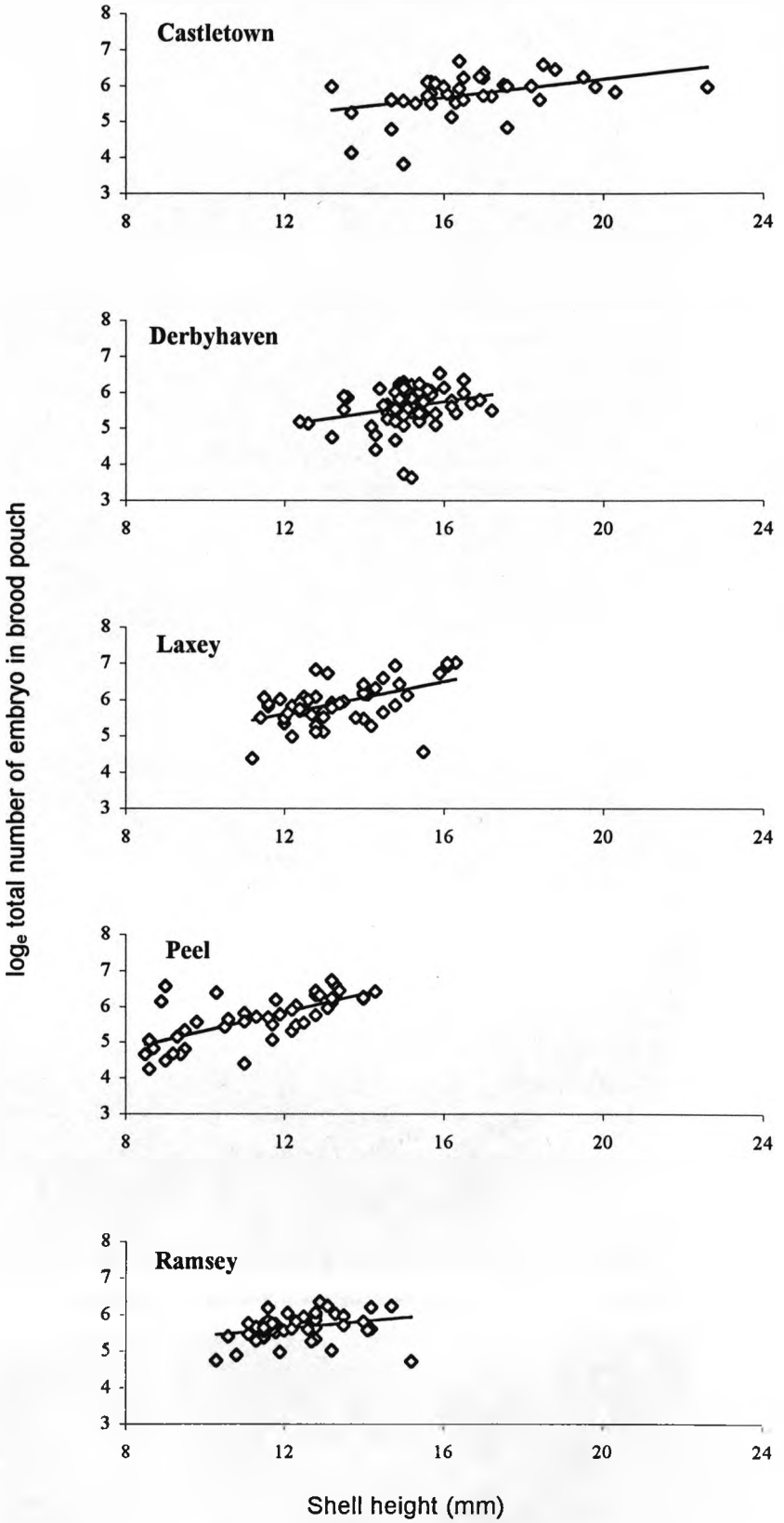

Fig 7.7 Regressions of log$_e$ total number of embryo in brood pouch against shell height of *Littorina saxatilis* from five sites in the Isle of Man collected in early July 1996. See regression equations in Table 7.5b.

Table 7.5 Equations and statistics for regressions of $\log_e$ total number of embryo in brood pouch against shell height (mm) in *Littorina saxatilis* from various sites in the Isle of Man. a = May, 1995 samples (see Fig. 7.5), b = July 1996 samples (see Fig. 7.6)

(a) Regression equations May 1995 samples

Site	n	$\log_e$(TNE) =	$\log_e$(a) +	b* Shell height	r^2 (%)	F-value	p<
Castletown	45	$\log_e$(TNE) =	2.61	0.190*Shell height	26.1	15.15	0.0001
Derbyhaven	84	$\log_e$(TNE) =	3.87	0.115*Shell height	4.9	4.19	0.05
Laxey	45	$\log_e$(TNE) =	2.71	0.251*Shell height	47.1	38.26	0.0001
Peel	73	$\log_e$(TNE) =	3.07	0.193*Shell height	18.2	15.78	0.0001
Ramsey	44	$\log_e$(TNE) =	3.20	0.199*Shell height	45.2	34.58	0.0001

TNE = Total number of embryo

(a) Regression equations July 1996 samples

Site	n	$\log_e$(TNE) =	$\log_e$(a) +	b* Shell height	r^2 (%)	F-value	p<
Castletown	39	$\log_e$(TNE) =	3.62	0.128*Shell height	17.3	7.73	0.01
Derbyhaven	63	$\log_e$(TNE) =	3.11	0.165*Shell height	7.2	4.73	0.05
Laxey	52	$\log_e$(TNE) =	2.95	0.222*Shell height	25.4	17.01	0.0001
Peel	46	$\log_e$(TNE) =	2.72	0.260*Shell height	48.4	41.31	0.0001
Ramsey	50	$\log_e$(TNE) =	4.43	0.099*Shell height	9.0	4.76	0.05

Table 7.6 Analysis of Covariance (ANCOVA) on regressions of $\log_e$ total number of embryo against shell height to test for size-specific differences in embryo numbers in *Littorina saxatilis* from various sites in the Isle of Man. Different analyses for samples collected in May 1995 and July 1996.

ANCOVA on May 1995 samples

Source of Variation	df	Seq SS	Adj SS	Adj MS	F	P
Site	4	35.196	0.593	0.148	0.59	0.673
Shell height	1	23.064	20.025	20.025	79.26	<0.001
SitexShell height	4	0.974	0.975	0.244	0.96	0.427
Error	281	70.996	70.996	0.253		
Total	290	130.231				

ANCOVA on July 1996 samples

Source of Variation	df	Seq SS	Adj SS	Adj MS	F	P
Site	4	3.122	0.944	0.236	0.98	0.418
Shell height	1	16.282	12.346	12.346	51.34	<0.001
SitexShell height	4	1.81	1.810	0.453	1.88	0.114
Error	240	57.72	57.715	0.241		
Total	249	78.93				

N/B significant interaction between site and shell height indicates a significant difference between slopes, site refers to intercept

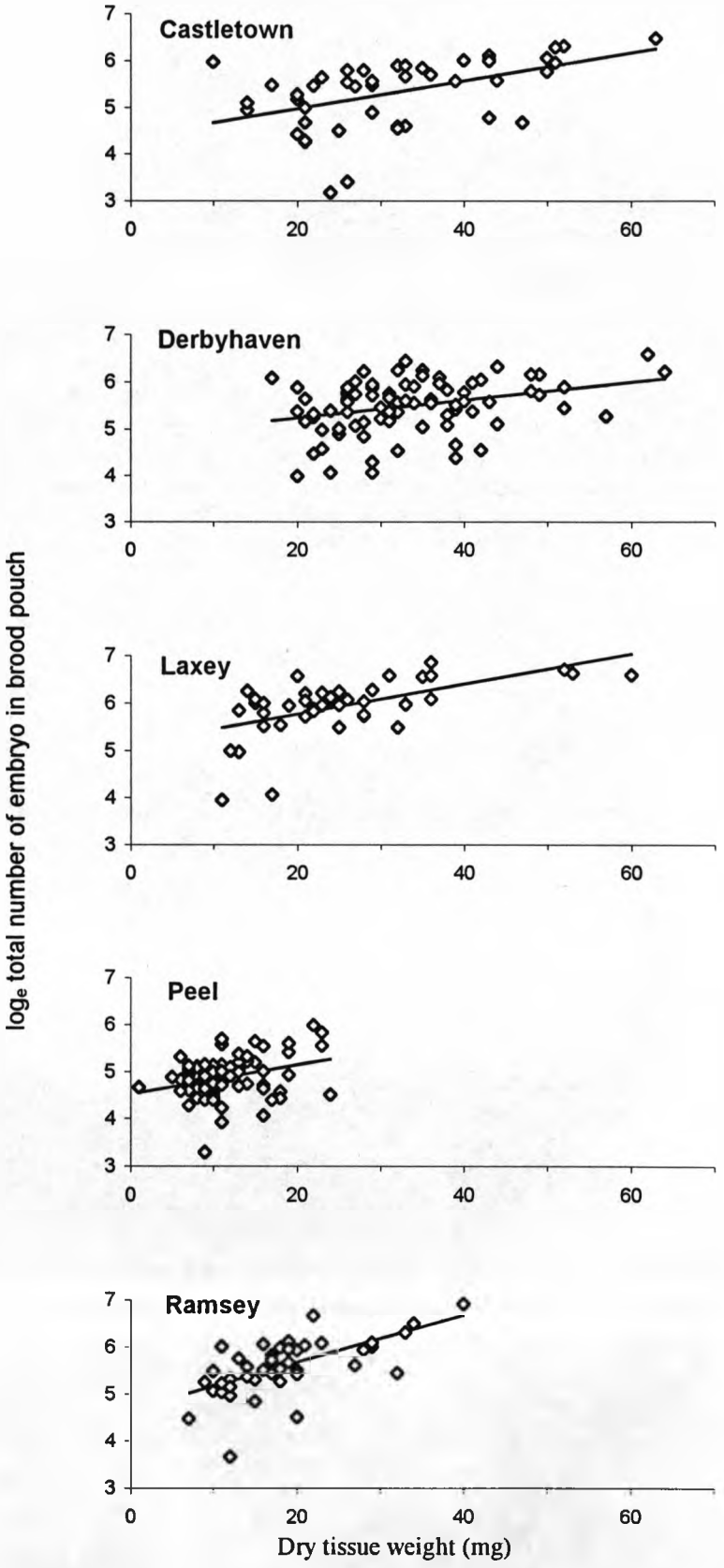

Fig 7.8 Regressions of $\log_e$ total number of embryo in brood pouch against dry tissue weight of *Littorina saxatilis* from five sites in the Isle of Man collected in May 1995. See regression equations in Table 7.7.

Table 7.7 Equations and statistics for regressions of $\log_e$ total number of embryo in brood pouch against dry tissue weight (mg) in *Littorina saxatilis* from various sites in the Isle of Man (see Fig. 7.7)

Site	n	$\log_e$(TNE) =	$\log_e$(a) +	b*weight	r^2 (%)	F-value	p<
Castletown	45	$\log_e$(TNE) =	4.37	30.0*weight	24	15.73	0.0001
Derbyhaven	84	$\log_e$(TNE) =	4.84	19.2*weight	10.8	9.95	0.01
Laxey	45	$\log_e$(TNE) =	5.13	31.7*weight	33.5	21.63	0.0001
Peel	73	$\log_e$(TNE) =	4.51	31.3*weight	10.2	8.02	0.01
Ramsey	44	$\log_e$(TNE) =	4.66	50.8*weight	40.1	28.11	0.0001

Table 7.8 Analysis of Covariance (ANCOVA) on regressions of $\log_e$ total number of embryo against dry tissue weight to test for weight-specific differences in embryo numbers in *Littorina saxatilis* from various sites in the Isle of Man.

Source of Variation	df	Seq SS	Adj SS	Adj MS	F	P
Site	4	35.196	2.330	0.582	2.22	0.068
weight	1	19.256	16.905	16.905	64.30	<0.001
Sitexweight	4	1.902	1.902	0.475	1.81	0.127
Error	281	73.877	73.877	0.263		
Total	290	130.231				

N/B significant interaction between site and weight indicates a significant difference between slopes, site refers to intercept

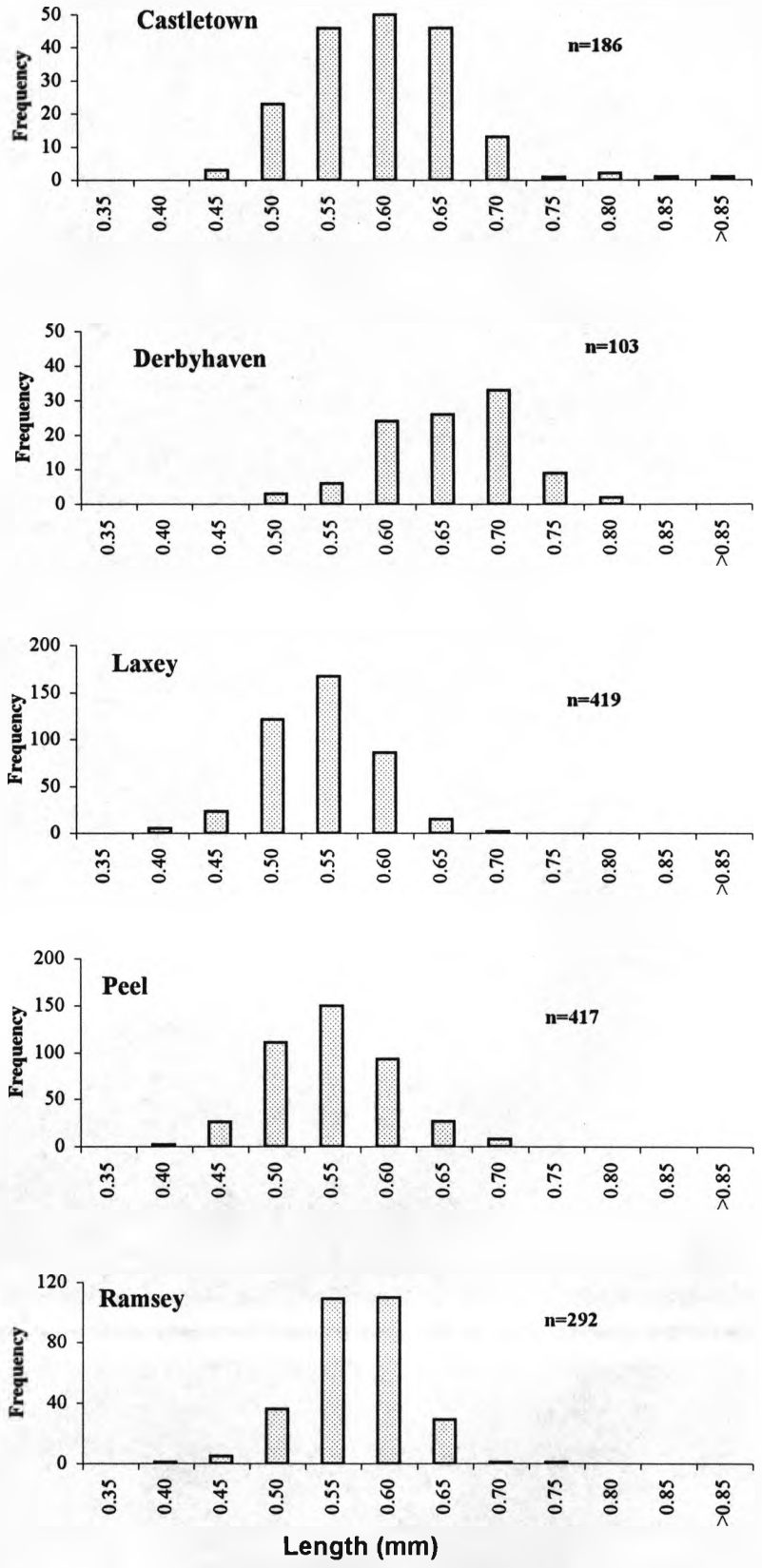

Fig 7.9 Length-Frequency histograms showing the size distribution of 0- to 24-hr juveniles of *Littorina saxatilis* from five sites in the Isle of Man.

Table 7.9 Size at birth (mm) of juveniles of *Littorina saxatilis* from five sites in the Isle of Man. Lowest mean size in bold

Size at birth of Juveniles

	n	minimum	maximum	mean	s.d
Castletown	186	0.405	0.876	0.574	0.071
Derbyhaven	103	0.457	0.776	0.632	0.063
Laxey	419	0.356	0.676	**0.517**	0.048
Peel	417	0.375	0.700	0.525	0.053
Ramsey	292	0.394	0.732	0.547	0.047

Table 7.10 One-way Analysis of Variance (ANOVA) and Tukey tests on differences in the mean size at birth of *Littorina saxatilis* from five sites in the Isle of man.

Source of Variation	df	SS	MS	F	P-value
Site	4	1.4174	0.3543	122.99	<0.0001
Error	1412	4.0681	0.0029		
Total	1416	5.4855			

Significance levels of Tukey test on mean size at birth

	Derbyhaven	Castletown	Ramsey	Peel
Castletown	***			
Ramsey	***	**		
Peel	***	***	**	
Laxey	***	***	***	ns

*=p<0.05, **=p<0.01, ***=p<0.001, ns=p>0.05

7.3.3 Embryo abnormality

The percentage of females with abnormal embryos was very high, ranging from 92 % in samples from Castletown to 100 % in those from Peel and Ramsey. The percentages of females with abnormals from Derbyhaven and Laxey were 98 % and 94 % respectively.

The proportions of abnormal embryos associated with shelled and unshelled stages of development are presented in Fig 7.10. Much of the abnormalities in animals from all sites were found in the unshelled embryonic stages. Individuals from Derbyhaven had by far the highest proportion of unshelled abnormals while those from Peel had the least. Animals from the metal contaminated Laxey site showed intermediate unshelled abnormalities. The mean proportions of shelled abnormalities were similar in all sites. There was a very high intra-site variability in the proportion of abnormals. Coefficients of variation ranged between nearly 100 % to well over 200 % (see Table 7.11). The Kruskal-Wallis test showed a significant difference in median unshelled abnormalities between sites (Table 7.12a); with individuals from Derbyhaven showing significantly higher abnormalities than those from all other sites (Dunn test, Table 7.12a). No significant differences were found for shelled abnormals between sites (Table 7.12b).

Fig. 7.11 shows the frequency distributions of the proportions of total embryonic abnormality in *L. saxatilis* from the five sites. Animals from Derbyhaven had the highest proportions of total abnormality. The Kruskal-Wallis test showed a significant difference in median total abnormalities between sites (Table 7.12c) and Dunn tests found a significantly higher abnormality in animals from Derbyhaven in comparison

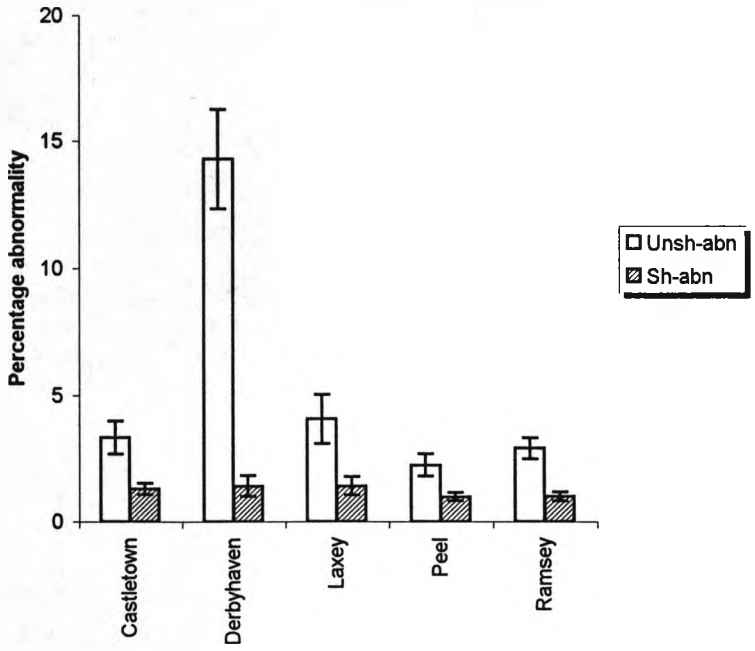

Fig. 7.10 Proportions (mean ± s.e.m.) of abnormal embryos associated with shelled and unshelled stages of embryonic development in *Littorina saxatilis* from various sites in the Isle of Man.
Unsh-abn = unshelled abnormals
Sh-abn = shelled abnormals

Table 7.11 Proportions of abnormal embryos (unshelled, shelled and total abnormals) in *Littorina saxatilis* from five sites in the Isle of Man. N/B large coefficients of variation.

(a) Unshelled abnormals

Site	n	minimum	maximum	mean	C.V. (%)
Castletown	39	0	23.70	3.34	120.95
Derbyhaven	63	0	55.95	14.31	108.39
Laxey	52	0	38.54	4.06	170.74
Peel	46	0	16.04	2.24	131.10
Ramsey	50	0.36	12.64	2.91	100.79

(b) Shelled abnormals

Site	n	min	max	mean	C.V. (%)
Castletown	39	0	5.16	1.30	108.52
Derbyhaven	63	0	16.67	1.41	232.94
Laxey	52	0	13.22	1.41	188.70
Peel	46	0	5.00	0.99	117.78
Ramsey	50	0	6.03	1.00	124.09

(c) Total abnormals

Site	n	min	max	mean	C.V. (%)
Castletown	39	0	27.27	4.64	106.11
Derbyhaven	63	0	57.54	15.99	104.62
Laxey	52	0	48.96	5.47	171.54
Peel	46	0.33	16.04	3.23	99.72
Ramsey	50	0.36	18.10	3.90	99.93

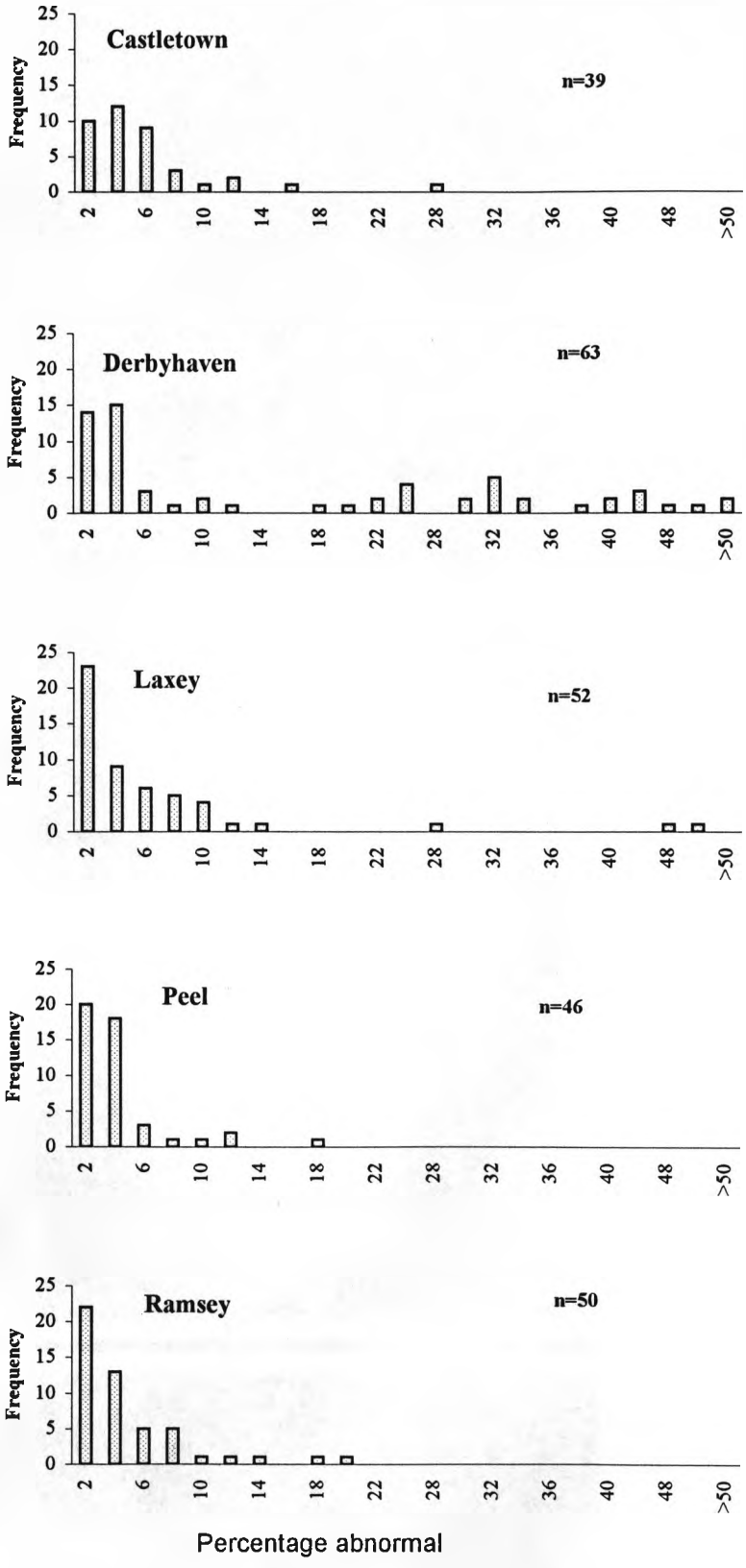

Fig. 7.11 Percentage abnormality-frequency histograms showing the distribution of total embryo abnormalities in *Littorina saxatilis* from five sites in the Isle of Man.

Table 7.12 Kuskal-Wallis One-way Analysis of Variance (ANOVA) and Dunn test on mean ranks to test for differences the proportions of abnormal embryos in *Littorina saxatilis* from five sites in the Isle of Man. Levels of significance of Dunn test *=p<0.05, **=p<0.01, ***=p<0.001, ns=p>0.05

(a) Kruskal-Wallis on % Unshelled abnormals

Site	n	Median	Mean rank
Castletown	39	2.551	123.8
Derbyhaven	63	3.627	168.4
Laxey	52	1.639	111.1
Peel	46	1.379	93.6
Ramsey	50	1.889	117.1
Total	250		125.5

H=33.87 (adjusted for ties) df=4, p<0.0001

Significance levels of Dunn test on unshelled abnormals

	Derbyhaven	Castletown	Ramsey	Laxey
Castletown	*			
Ramsey	**	ns		
Laxey	***	ns	ns	
Peel	***	ns	ns	ns

Kruskal-Wallis on % Shelled abnormals

Site	n	Median	Mean rank
Castletown	39	0.756	142.5
Derbyhaven	63	0.435	113.1
Laxey	52	0.575	127.5
Peel	46	0.582	126.5
Ramsey	50	0.661	124.8
Total	250		125.5

H=4.16 (adjusted for ties), df=4, P=0.386
No significant difference

(c) Kruskal-Wallis on total abnormals

Site	n	Median	Mean rank
Castletown	39	3.333	128.5
Derbyhaven	63	5.195	162.7
Laxey	52	2.029	108.7
Peel	46	2.291	104.0
Ramsey	50	2.577	113.5
Total	250		125.5

H=25.02 (adjusted for ties), df=4, p<0.0001

Significance levels of Dunn test on total abnormals

	Derbyhaven	Castletown	Ramsey	Laxey
Castletown	ns			
Ramsey	**	ns		
Laxey	***	ns	ns	
Peel	***	ns	ns	ns

with those from Ramsey, Peel and Laxey. However, no significant difference in median total abnormality was found between animals from Derbyhaven and those from Castletown.

The relationship between shell height and total proportions of abnormals is presented as scatter plots in Fig. 7.12. Spearman's rank correlation coefficients indicated that no significant correlation existed between shell height and the proportion of abnormals at any of the sites studied.

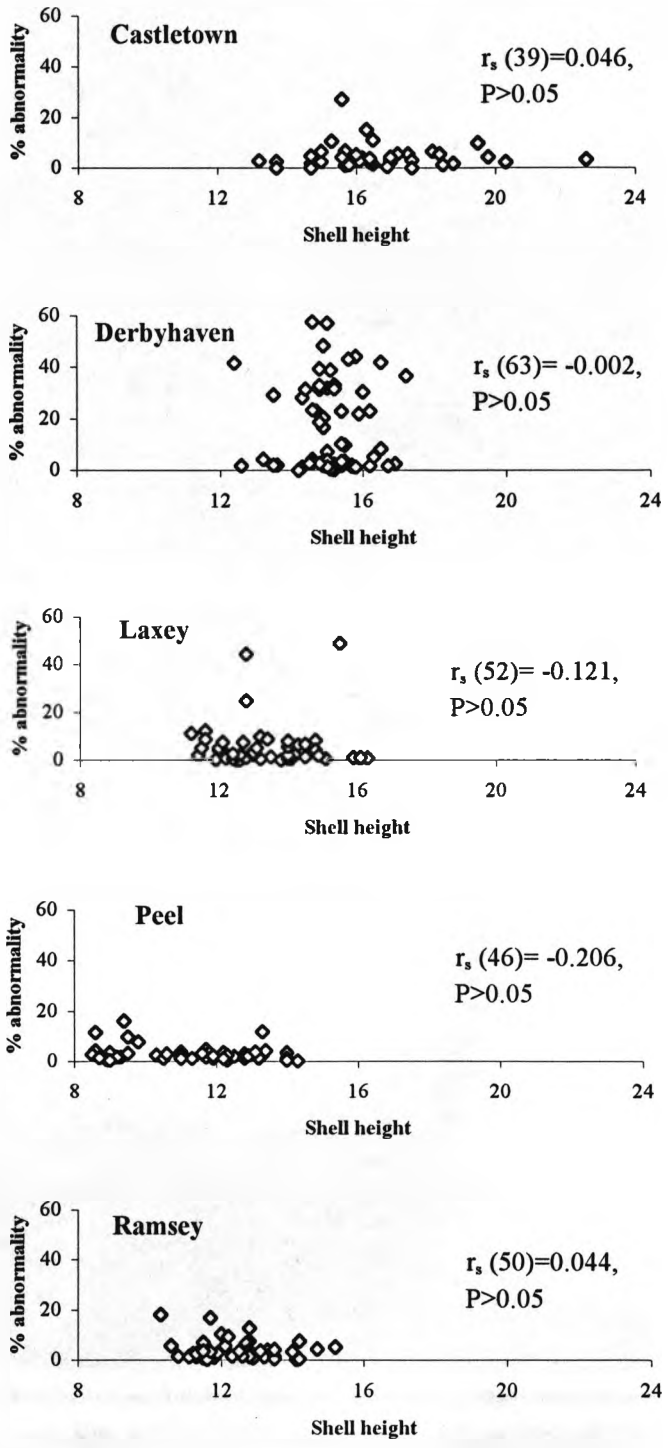

Fig. 7.12 Relationship between shell height and percentage embryo abnormality in *Littorina saxatilis* from five sites in the Isle of Man. r_s = Spearman's rank correlation coefficient.

7.4 DISCUSSION

7.4.1 Fecundity and size at birth of juveniles

Intraspecific differences in numbers of embryo and size of juveniles at birth in *Littorina saxatilis* has been reported to vary with the level of exposure of the shore from which samples are collected (e.g. Faller-Fritsch, 1977; Roberts & Hughes, 1980, Janson, 1982). Such variation will have to be taken into account when interpreting the likely effects of pollution on these reproductive parameters. Conflicting reports have been found in the literature with respect to how exposed/sheltered shore types affect these reproductive parameters.

Janson (1982) measured the size of new born juveniles from a Swedish coast and compared the sizes of newly born juveniles of two morphs, exposed (E-morph) and sheltered (S-morph) from populations only 50 metres apart. Janson found that despite being smaller, E-morph females on the average gave birth to larger juveniles than did S-morph females; and concluded that this difference in hatching size is likely to be of genetic origin and an aspect of the adaptation to the environment. Larger juveniles are favoured in an exposed habitat because of a higher adhering capability of the snail which is proportional to the foot area while a larger number of smaller offspring could be a better parental investment in sheltered areas. Faller-Fritsch (1977) wrote "a given reproductive energy expenditure may involve large numbers of small embryo or fewer larger embryos. Preliminary investigations of *Littorina rudis* (= *L. saxatilis*) in many parts of Britain support the view that these differences are general in occurrence, larger embryos being characteristic of sheltered populations". The two authors have made generalisations of a fundamental nature with respect to the effect of shore

exposure on embryo size at birth and potential reproductive output which are diametrically opposite.

Roberts and Hughes (1980) studied fecundity and size of embryo at birth in *L. rudis* (=*L. saxatilis*) from three Welsh sites, a sheltered saltmarsh, a semi-sheltered boulder shore and an exposed cliff. They found that the largest juveniles were produced by the population from the most sheltered site. However, the smallest juveniles were not obtained from the exposed shore but rather from the semi-sheltered boulder shore and they did not find any significant differences in the size-specific number of embryo in the brood pouch. They concluded that "it seems unlikely that there is a significant trade off between the size of offspring at birth and parental fecundity in *L. rudis*. Inter-population variations in size at birth may result from selection pressure on parental investment per offspring, caused by such factors as parental versus juvenile survivorship and competition among juveniles".

The interpretation of the effects of pollutants on fecundity and embryo size at birth against such a backdrop of apparent uncertainty in natural trends is difficult. The locations from which samples were collected for this work were essentially sheltered, efforts having been made to control for the effect of exposure. However, some differences were apparent in the degree of exposure of the locations from which samples were taken with Peel and Ramsey being slightly more exposed, the former being more so than the latter. Overall, Castletown and Derbyhaven were the most sheltered and Laxey was intermediate between the above four sites. The size structure

of adults at these sites reflect the above pattern, the animals from Peel being the smallest and those from Castletown the largest.

If one were to explain the differences in juvenile size and reproductive output strictly on the basis of site exposure/shelter, the intermediate level of Laxey would have meant that values somewhere within the two "extremes" would be obtained. This was not the case as, *L. saxatilis* from Laxey had the smallest mean embryo size and the highest mean absolute number of embryos both in May and July as well as the highest size-specific brood pouch content in May. The situation of the animals from Laxey does not also conform to the contention by Roberts and Hughes (1980) that there is no trade-off between size at birth and fecundity, since, the lowest size at birth coincided with the highest fecundity at least in May.

A likely explanation for the size at birth and fecundity of *L. saxatilis* at Laxey relative to the snails from other sites is exposure to high levels of heavy metals, mainly Zn. The effects of heavy metal on the size at birth may be direct or indirect. The higher level of heavy metal exposure may directly cause a reduction in the size of embryo. If that is the case the larger number of embryos is probably a response to counter-balance the increased mortality that may be associated with small-sized juveniles. On the other hand the smaller sized individuals may be an indirect consequence of the need to produce large numbers of embryo in response to high metal-related juvenile mortality. Bodar *et al.* (1988) observed a similar response in *Daphnia magna* exposed to Cd in laboratory experiments. Cd concentrations of 0.5, 1.0 and 5.0 ppb were found to significantly increase the average number of neonates per female, but the size of the neonates were smaller. They found, however, that at higher concentrations both brood

size and body size declined. The fact that seasonal effects can modify the relative differences in size-specific numbers of embryo requires that further studies be conducted, incorporating seasonal effects to give a more complete picture. Also the general problem of whether animals from sheltered shores produce larger juveniles or not needs to be resolved on a broad scale. Studies of animals from a large number of sites with different exposure scales (and possibly geographical locations) need to be made.

Other factors which may affect the differences in embryo numbers *L. saxatilis* are the availability of food and the relative period of tidal exposure which could affect foraging time (Faller-Fritsch, 1977). Berry (1956) observed that fecundity in snails was higher for individuals collected from the upper shore than those from the lower shore.

7.4.2 Embryo abnormality

The results of this study did not show any metal-related increase in the proportion of abnormal embryos in the Isle of Man populations of *Littorina saxatilis*, but this may not always be the case. Dixon & Pollard (1985) studied the differences in the frequency of abnormal embryos of in *L. saxatilis* from the southern coast of Wales. They found that individuals from a site which was furthest away from the known sources of pollution had the lowest frequency of abnormal embryos in comparison with those from other sites. The pollutants in the area studied by Dixon & Pollard include some which had mutagenic or teratogenic effects (capable of inducing abnormal development of embryos) derived from diverse sources. These include heavy metals (Ni, Hg, Cd), petroleum hydrocarbons and organochlorine pesticides such as gamma

HCH, lindane and dieldrin. It is not entirely surprising that the snails from Laxey did not have any high proportion of abnormals because Zn which is the most elevated metal in that site is not known to have any teratogenic or mutagenic effects (BDH hazard assessment handbook). Dixon and Pollard (1985) did not find any correlation between the gradient of contamination and frequency of abnormality and they suggested that a complex aetiology might be involved. It is not unlikely that the animals from Derbyhaven experience some form of contamination that has not been detected in this work. The site is close to the Ronaldsway airport and industrial complex, and outfalls of untreated sewage. There is probably contamination from sources other than the heavy metals monitored in this study, the confirmation of which would show that embryo abnormality examination is a good tool for detecting pollution.

Another example where environmental stress has been found to increase the level of embryo abnormality in *L. saxatilis* is in response to low salinity. While Thorson (1946) and Sokolova (1995) found high proportions of abnormality in lower salinities in samples from Φresund sound (Denmark) and White Sea populations respectively; neither Clyne and Duffus (1979) (for samples from the north coast of Scotland) nor Janson (1985) (for snails from the Swedish coast) found such an effect. Sokolova (1995) reported that the proportions of abnormal embryos and abnormal juveniles correlated with decreasing salinity but that did not apply to the proportion of abnormal eggs which was highest at intermediate salinity, again indicating that salinity could not entirely account for the changes observed. No salinity variations occur at Derbyhaven where the population with the highest proportions of abnormal embryos

were found in this study. This rules out salinity as a possible cause of the high frequency of abnormality at that site.

Much of the abnormal embryos recorded in this study were found to be associated with unshelled stages of development. Dixon & Pollard (1985) and Sokolova (1995) also obtained similar results. In the opinion of the latter author, the decrease in the proportion of abnormals from earlier to later stages of development might suggest strong selection against abnormals during early ontogenesis.

Dixon and Pollard (1985) suggest that abnormalities may be caused by a latent pathogen which is activated to disease condition by environmental stress. They reported that physical features of the most dominant type of abnormality in the environmentally stressed populations of periwinkles were reminiscent of the kind of uncontrolled breakdown in the processes associated with normal growth and development. This might be expected from a neoplastic disorder, in this case confined within the egg capsule. However, they did not find any pathological condition to be associated with the somatic tissue of adult snails containing high incidence of abnormality; and neither did electron micrographs show any evidence of virus particles in the cells of abnormal embryos. The predominant type of embryo abnormality observed in *L. saxatilis* from Derbyhaven bear the above features described by Dixon & Pollard (1985). It is likely that some disease condition (not necessarily triggered off by stress) is responsible for the high incidence of abnormalities at that site.

Janson (1985) argues that genetic factors may play an important role since no environmental stress factors unquestionably influence the degree of embryo

abnormality. Large within-site variations (and probably less significant between-site variations) in embryo abnormality were suggested to be due to genetic rather than environmental factors. Presumably, inbreeding in small random mating groups was the primary genetic factor involved. The possibility of such an effect occurring in the Isle of Man populations of *L. saxatilis* cannot be ruled out as very high coefficients of variation were found in the proportions of abnormals in these sites.

No significant correlations were found between shell height of female snails and percentage embryo abnormality in this study. Sokolova (1995) similarly found that the ages of females determined from distinct growth rings, found in the White Sea populations, had no influence on the proportion of abnormal embryos. Crepede (1910, cited in Janson, 1985) suggested that ciliate infection increases the risk of embryo abnormality. However, Dixon and Pollard (1985), and Sokolova (1995) did not find any differences in the proportions of abnormals between uninfected females and those parasitized with the ciliate (*Protophyra avicola*). My observations were also that ciliate infection did not increase the proportion of abnormals although this was not quantitatively assessed.

In conclusion, contamination by heavy metals may be exerting a direct or indirect effect leading to a reduction in the size of embryo at birth in *L. saxatilis* from Laxey. The relatively high number of embryo in the brood pouches of the snails from Laxey is likely to be an adaptive response to mitigate the effects of metal-related juvenile mortality. No metal-induced embryo abnormalities were apparent in this study. The high proportions of abnormal embryos in animals from Derbyhaven which are not

known to be exposed to high levels of heavy metal pollution may be due to disease and/or genetic factors. Whether unidentified chemical contaminants from the nearby industrial complex has caused the observed high frequency of abnormality in the individuals from Derbyhaven is unknown.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

8.1 GENERAL DISCUSSION

In the preceeding chapters, I have described field and laboratory studies with *Littorina saxatilis* that could have a bearing on the use of this species as a biomonitor organism for heavy metals, as well as giving evidence of impact of long-term heavy metal contamination. This chapter is a brief overview of the limitations of the work and the problems and prospects raised by the results obtained.

Littorinids are known to have the ability to regulate certain metals especially those that are essential (e.g. Zn, Cu and Fe) and this is a draw-back in their use as monitors for these metals (Young, 1975, Bryan *et al.*, 1983, Bryan, 1979; Webb, 1990). The regulation of Cu in *L. saxatilis* was confirmed in this work as Cu levels in the animals showed no correlation with levels in *Enteromorpha*. Zn appeared to be less regulated and Cd was significantly correlated with values in the algae. The development of tolerance and the interactions between metals may also affect the use of *L. saxatilis* as a biomonitor.

Tolerance has both positive and negative aspects in relation to biomonitor ability. On one hand it is an important requirement that an organism for use as a biomonitor should be able to withstand the levels of pollutants encountered in the environment (Phillips, 1980). However, when long-term exposure to metals in a population has resulted in the development of metal tolerance, the tissue burdens of animals collected for analysis may not necessarily reflect metal availability. Depending on the most important mechanism by which tolerance is achieved, either excessive or lower tissue burdens might be found. Winkles from Laxey have been subjected to a long-term

pollution from mining activity spanning a period of over two centuries. Although mining stopped over 70 years ago, contaminant delivery persists and the animals from Laxey have been shown to have an enhanced tolerance to acute exposures to Zn and Pb in the laboratory. The long period over which the animals have been exposed to metals could possibly have led to genetic selection for metal tolerance (discussed further below).

Interactions between metals may also affect the tissue concentrations of metals. In laboratory accumulation experiments, Zn was observed to have an antagonistic effect on Pb and Cd accumulation. These experiments were conducted at high metal concentrations which may not be environmentally realistic and the concentration of Zn was higher than other metals in all the combinations. However, such an interaction may be capable of reducing the levels of the antagonised metals in the field. Interactive effects between metals may change with changes in the metal concentrations (see Phillips & Rainbow, 1993); how such a change would affect the observed interactions is unknown. Furthermore, responses targeted at one metal could reduce the accumulation of another metal. The detection of Cu in solution at 1mg l^{-1} Cu was found to result in a reduction of Zn accumulation. Whether Cu will have similar effects on other metals such as Pb and Cd was not investigated and could be a subject for further study.

Environmental pollutants may exert strong selective pressures on natural populations, offering unique opportunities for studying natural selection (Klerks & Levinton,1989). Long-term effects of pollutants on natural populations will be very different from those predicted from bioassays if populations inhabiting polluted sites commonly evolve a

resistance to the toxicants present. The presence of a genetic basis of metal tolerance has been reported for bacteria (O'Halloran & Walsh, 1987), marine algae (Russell & Morris, 1970) and terrestrial plants (Bradshaw, 1952; Antonovics *et al.*, 1971; see Bradshaw & McNeilly, 1981 for reviews). Evidence for the genetic basis of heavy metal tolerance in aquatic organisms can be found in the tolerance of *Asellus meridianus* from the Gannel (Brown, 1976). She found that tolerance to Pb in that species persisted in F_1 generation of animals reared in metal-free environments. Circumstantial evidence also suggests that tolerance to Cu in *Nereis diversicolor* from Restronguet creek is genetically determined (Bryan & Hummerstone, 1971, Bryan & Gibbs, 1983); worms reared in the laboratory exhibited similar levels of tolerance to those collected freshly from the wild and these were higher than control animals (freshly collected or reared). Klerks & Levinton (1989) found rapid evolution to a mixture of metals (Cd, Ni and Co) in the olygochaete *Limnodrilus hoffmeisteri* from metal polluted sites in Foudry Cove (New York). Webb (1990) did not find any genetic basis for Zn tolerance in *L. saxatilis* from Dulas Bay. However, his experiments were only preliminary and involved an examination of differential uptake of Zn in animals from Dulas Bay in comparison with uptake rates by animals from nearby control sites. Apart from the fact that metal uptake was examined at only one Zn concentration, the use of differential uptake rate is not a conclusive end-point for assessing tolerance. Acute toxicity tests need to be performed using F_1s to be able to conclusively determine whether tolerance can be genetically determined or not. In the case of the populations from Laxey where exposure to metals has spanned such a long period, it is possible that metal tolerance could have a genetic basis. Attempts were made to rear animals in the laboratory using the method of Warwick (1982) but

successive cultures of juveniles recorded huge mortalities after a few months and it was not possible to produce sufficient animals for experimentation. One reason for the failure may be that the quality of food provided was insufficient as growth was very slow before the mortality events occurred.

Bryan (1976b) wrote "From the point of view of the organism and the polluters, the ability to adapt to metals seems to be a good thing. However, it must be remembered that these tolerant organisms contain two or three orders of magnitude higher metals than normal and, so far as we know at present, these may be transmitted to non-adapted predators, including man". Recent evidence suggests that much of the potentially high dietary metal is in detoxified form. For example, barnacles which are very strong accumulators of Zn, store this metal in the form of Zn pyrophosphate granules (Rainbow, 1987; Pullen & Rainbow, 1991). Nott & Nicolaidou (1990) found such granules in the faecal pellets of *Nucella lapillus* feeding on barnacles, indicating that the Zn is not in fact available to the consumer. Regardless of the imminent danger of effects at higher trophic levels, and the inherent advantage of the dependence of the survival of individuals of a species depending on the development of tolerance in certain heavily polluted areas; the question may be asked as to whether there are any "trade-offs" due to metal tolerance. Viarengo *et al.* (1995) found that superimposing tolerance to a general stressor on metal stressed individuals of *Mytilus* resulted in higher susceptibility in metal pre-exposed individuals. Webb (1990) observed that *L. saxatilis* from contaminated sites which showed tolerance to low but toxic concentrations of Cu and Zn suffered higher mortalities to salinity and desiccation stress. My results also showed that animals from Laxey might exhibit a cost of tolerance to intertidal stress, especially desiccation. The interpretation of the results

was made difficult by the effects of natural factors such as shore height and salinity regime on the results obtained.

Pollution at sub-lethal levels which may seemingly have no effect on adult populations may exert reproductive effects (Åkesson, 1983, Dixon & Pollard, 1985). One problem raised by the use of reproductive parameters in *Littorina saxatilis* to estimate the impact of heavy metals in the field is the standardisation of sampling units as a result of inter-site differences in growth rate and possible reproductive periodicity. The degree of exposure of shore and shore height could affect the growth rate of animals such that one is faced with comparing reproductive output in populations with different size structures. It can be reasonably assumed that larger sized individuals will have a greater capacity for accommodating a larger number of individuals in the brood pouch but how the allocation of energetic requirement to somatic growth will affect the reproductive output is not known. Individuals of a standard shell height (e.g. Faller-Fristch, 1977) may be compared. Alternatively, individuals in the range of sizes of reproductive age could be used (e.g. Janson, 1985, this work) and size specific numbers of brood pouch compared by regressing counts on shell height. Both of these approaches assume a functional relationship between shell height and number of embryo. The very low regression coefficients obtained in this work show that shell height accounts for a minimal proportion of the variation in brood pouch content. This could seriously have affected the results obtained.

Although the brood pouches of *L. saxatilis* may contain embryos throughout the year (Hannford Ellis, 1983), there might be seasonal variations in peak reproductive activity (Berry, 1956. Faller-Fritsch, 1977) which may vary with locality and could have

affected the results. One might compare a population with a high proportion of reproductively active females with another where reproductive activity is low at the time of sampling. I found appreciable variation in reproductive output in animals from Peel and Castletown but those from Laxey, Derbyhaven and Ramsey had similar absolute brood pouch contents in the two sampling times (May 1995 and July 1996). The variation could possibly be annual. A higher frequency of sampling, possibly monthly over a year, could be made and reproductive output estimated on an annual basis for comparison. Such an approach could not be used for this work because of limitations of time.

8.2 FURTHER WORK

Some suggestions for further work have been made in the preceding discussion. One important area for emphasis is the basis of the observed tolerance to acute metal levels in *Littorina saxatilis*. Whether tolerance to Zn and Pb represent a mere expression of phenotypic plasticity or have been genetically selected for in the animals from Laxey needs to be conclusively assessed. The most appropriate approach would be to rear animals from contaminated and control sites in a metal-free environment in the laboratory and to test for tolerance to metals in F_1s. The molecular mechanisms by which animals have become genetically adapted to metals may also be studied. Even if a genetic basis of tolerance were not to be proved, the molecular mechanisms involved in the physiological adaptations would make an interesting study. A variety of environmental stressors have been shown to induce alterations in gene expression and

the gene products may have a role in cellular protection (Glaven *et al.*, 1993). Gene expression in *L. saxatilis* from the metal contaminated site could be studied.

8.3 CONCLUSIONS

1. Contamination of Manx esturaries associated with erstwhile mining areas persists and was detected in very high levels of Zn and Pb in sediment and in the tissues of the biomonitors, *Mytilus edulis, Fucus serratus, Littorina saxatilis and Enteromorpha intestinalis*.

2. *L. saxatilis* from the Laxey estuary has developed an enhanced tolerance to acute levels of Zn and Pb with the tolerance to the two metals seemingly independent. No co-tolerance to Cu or Cd was observed.

3. Metal tolerance appears to have resulted in some physiological trade-off as the tolerant animals from Laxey were generally less tolerant to desiccation stress. Tolerance to extreme salinities appeared to be unaffected.

4. There were different mechanisms involved in the tolerance of Zn and Pb. While Zn tolerance was associated with lower rate of Zn accumulation, Pb tolerant individuals took up significantly more Pb than non-tolerant animals.

5. Cu accumulation in *L. saxatilis* was erratic and was not affected by the presence of Zn in solution. However, Zn had an antagonistic effect on the accumulation of Cd and Pb at the experimental concentrations used.

6. Heavy metal contamination in Laxey has resulted in some reproductive effects in *L. saxatilis* and this was reflected in a reduced size at birth of young. No metal-related increase in the frequency of embryonic abnormality was observed.

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Appendix A. Comparison of metal concentations measured in sediment and biological reference material with values certified by the Community Bureau of Reference (BCR). Measured values were within ± 10% of certified values, except for one instance where cadmium was slightly higher.

Appendix A1 Sediment Analysis

	Conc. $\mu g g^{-1}$		
Metal	Measured	Certified	% difference
Zinc	570.72	547.00	4.16
Lead	151.55	146.00	3.66
Copper	93.73	101.70	-8.50
Cadmium	13.20	11.90	9.85
Iron	-----	-----	-----

No certified values available for iron

Apendix A2 Biomonitor analyses

Myilus edulis ref. in *M. edulis* analysis

	Conc. $\mu g g^{-1}$		
Metal	Measured	Certified	% difference
Zinc	72.73	76.00	-4.49
Lead	2.12	1.91	10.01
Copper	10.57	9.60	9.15
Cadmium	0.38	0.34	9.91

Mytilus edulis reference in *Littorina saxatilis* analysis

	Conc. $\mu g g^{-1}$		
Metal	Measured	Certified	% difference
Zinc	76.18	76.00	0.24
Lead	2.10	1.91	9.00
Copper	8.75	9.60	-9.72
Cadmium	0.37	0.34	8.41

Ulva lactuca reference in *Fucus serratus* analysis

	Conc. $\mu g g^{-1}$		
Metal	Measured	Certified	% difference
Zinc	49.01	51.30	-4.68
Lead	13.95	13.48	3.37
Copper	12.33	13.14	-6.54
Cadmium	0.300	0.274	8.77

Ulva lactuca reference in *Enteromorpha intestinalis* analysis

	Conc. $\mu g g^{-1}$		
Metal	Measured	Certified	% difference
Zinc	55.39583	51.3	7.39
Lead	14.7375	13.48	8.53
Copper	12.46667	13.14	-5.40
Cadmium	0.316667	0.274	13.47

Appendix B. Median lethal times (bold) or maximum mortality of *Littorina saxatilis* in toxicity tests where less than 50 % mortality was observed in some repicates/blocks at the termination of experiments.

5 μgl^{-1} Zn

Replicate/ Block	Castletown	Derbyhaven	Laxey	Peel	Ramsey
1	**18**	**24**	20	**17.4**	**13**
2	**16**	**11**	40	**16**	**10.9**
3	40	20	10	**16**	**28**
4	40	40	10	**40**	**20**
5	**40**	30	10	**20**	**24**

5μgl^{-1} Pb

Replicate/ Block	Casletown	Derbyhaven	Laxey	Peel	Ramsey
1	**19.3**	**24**	**38.7**	**24**	**24**
2	**22.7**	**29**	**32**	**23.3**	**29.4**
3	**25**	**30**	30	**26**	**32**
4	**24**	**30**	20	**26**	**28**
5	**21.6**	**26.5**	**38.7**	**22**	**26**

0.5 μgl^{-1} Cd

Replicate/ Block	Casletown	Derbyhaven	Laxey	Peel	Ramsey
1	40	30	40	10	**28**
2	**25**	40	**28**	**26.3**	40
3	**28**	0	40	20	40
4	40	0	10	0	10
5	0	0	10	20	30