The Role of a Symbiotic Bryozoan in the Chemical Ecology of a Marine Benthic Predator – Prey Interaction

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The South African west coast rock lobster, *Jasus lalandii*, and the bryozoan encrusted whelk, *Burnupena papyracea*, on the mussel and urchin dominated benthos off Cape Town, South Africa.

"The only solid piece of scientific truth about which I feel totally confident
is that we are profoundly ignorant about nature."
Lewis Thomas, from *The Medusae and the Snail*, 1979

Dedicated to my parents, Alan and Larraine,
with love and admiration.

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Abstract

The subtidal whelk *Burnupena papyracea* (Brugière) co-occurs with a voracious predator, the rock lobster *Jasus lalandii* (Milne Edwards), in situations where other potential prey are largely eliminated. This has been ascribed to a symbiotic bryozoan, *Alcyonidium nodosum* (O'Donoghue and de Watteville), which characteristically encrusts the shells of *B. papyracea* and deters feeding by *Jasus*. In this study it is shown that this is not due to physical effects of either induced physical defences in the bryozoan or increased shell strength due to the presence of the bryozoan. Neither spectroscopic screening of chemical extracts of the bryozoan nor analysis for volatile constituents revealed any apparent chemical components that are likely to deter feeding. Chemical extracts also failed to show larvicidal effects in a standard toxicity assay using the brine shrimp *Artemia salina* (Leach). Despite this, bioassays using individual *Jasus* indicated a chemical basis for feeding deterrence.

The assays were run separately on three sets of *Jasus* and some repeats of assays gave contradictory results. However, assays showing no significant effect of treatment occurred with moulting *Jasus*, involved very low overall feeding rates and so gave a less convincing result. In other assays *Jasus* always avoided *Burnupena papyracea* with live *Alcyonidium* encrusting the shell, and food pellets containing *Alcyonidium* or an *Alcyonidium* extract. Significant preferences were shown for an unencrusted whelk, *B. cincta* (Röding), over *B. papyracea*; for *B. papyracea* with the bryozoan scraped off over natural *B. papyracea*; for *B. papyracea* on which the bryozoans had been killed with liquid nitrogen over untreated *B. papyracea*; and for food pellets prepared from ground, dried mussel over pellets prepared with dried mussel mixed *with A. nodosum* or its crude organic extract.

It is concluded that the protection which *Alcyonidium* confers on *Burnupena papyracea* does have a chemical basis, but that the chemical responsible is either present in only trace quantities, or that it is a structurally unremarkable compound which is distasteful to *Jasus*. This work highlights both the advantages of using ecologically relevant bioassays (positive results when standard techniques give a negative result) and also the disadvantages (logistic constraints on sample

sizes when using large test animals and individual variability in a relatively sophisticated test animal).

Chapter 1

General Introduction: Chemical Ecology

Ecology, in its broadest sense, is the study of the interactions of organisms and their environment (Smith, 1992). The environment experienced by any organism is determined by its physical and structural surroundings (abiotic factors) as well as by other organisms (biotic factors) present in the natural system (Kormondy, 1969; Putman and Wratten, 1984). The abiotic, physico-chemical properties of any ecosystem are perhaps the most obvious and fundamental aspect of the environment, as these are the primary factors which determine whether an organism can or cannot exist in a certain habitat (Putman and Wratten, 1984). Temperature, light intensity, degree of exposure to various physical elements (*e.g.* prevailing winds, wave-action, desiccation, *et cetera*), substratum characteristics and availability of essential elements *etcetera* are but a few important abiotic characteristics of any environment which exert a profound influence upon the organisms that inhabit it (Kormondy, 1969).

Within this structural framework, however, there exist more subtle, biotic interactions between the organisms living in a specific environment (Kormondy, 1969; Putman and Wratten, 1984). Territoriality, predation, parasitism, competition, commensalism and mutualism all play roles in determining the structure and dynamics of a particular ecosystem (Levinton, 1995). These interactions, which may be inter- or intra-specific, serve to modify an organism's existence within its physical surroundings further and ultimately determine the fine detail of its ecology (Putman and Wratten, 1984).

Extensive investigation of the ecology of terrestrial systems has indicated that many biotic interactions are in fact mediated by chemical agents (for examples see Agosta, 1996). Relatively recent innovations in techniques related to analytical chemistry, and conceptual advances in behavioural biology have helped to focus attention on the importance of chemical interactions in biological processes (Eisner and Meinwald, 1995). In this respect, research into the nature and role of the chemicals concerned with various ecological phenomena has evolved into the field of chemical ecology (Sondheimer and Simeone, 1970; Whittaker and Feeney, 1971) which has developed during the last century into a mature discipline on the interface of biology and chemistry (Eisner and Meinwald, 1995; Mitchel-Olds *et al*., 1998).

1.1. Chemical ecology: concepts, definitions and objectives

The role of chemical compounds in the ecology of terrestrial plants and animals has been well documented (see Sondheimer and Simeone, 1970; Harborne, 1999 and previous reviews in this series). This research has disclosed the surprising complexity and number of ways in which organisms utilise organic chemicals in ecological interactions (Brown *et al*., 1970; Sondheimer and Simeone, 1970; Whittaker and Feeney, 1971). In fact, chemically mediated interactions are now considered ubiquitous in biological systems (Eisner and Meinwald, 1995). Methods of chemical defence in particular have been observed in all phylogenetic kingdoms (Whittaker and Feeney, 1971) and chemical communication has been described as the paramount mode of interaction in most groups of animals (Wilson, 1970). It has become apparent that all organisms engender some form of chemical signal, or *semiochemical* (Gk. *simeon*, a mark or signal; Law and Regnier, 1971), and all respond to the chemical emissions of others (Eisner and Meinwald, 1995).

As with any developing area of science, a system of nomenclature has evolved to deal with the various concepts and chemical interactions observed in chemical ecology (outlined in figure 1.1). The term *pheromone* (Gk. *phereum*, to carry, and *horman*, to excite or stimulate), proposed by Karlson and Butenandt (1959) and Karlson and Lüscher (1959), is now well established and is commonly used to describe an external chemical signal employed in intra-specific communication. The nomenclature describing inter-specific communicative agents, or *allelochemics* (Whittaker, 1970), is much less familiar. Chemical compounds within this category may be differentiated into three groups: *Allomones* (Brown, 1968) are transpecific messengers which impart some adaptive advantage on the emitting species; *Kairomones* (Gk. *kairos*, opportunistic; Brown *et al*., 1970) are agents, the adaptive benefit of which falls on the recipient rather than the emitter; and *Synomones* (Gk. *syn*, with or jointly; Nordlung and Lewis, 1976) are substances which evoke a response adaptively favourable to both emitter and receiver.

It should be noted that the major categories of chemical messengers (pheromones, allomones, kairomones and synomones) are not mutually exclusive (Brown *et al*., 1970): research has shown that a great many compounds can be assigned two or three roles according to the above nomenclature because of the complex nature and number of interactions with which they are involved (Whittaker and Feeney, 1971; Nordlung and Lewis, 1976). Consequently, the definitions and terminology employed in the field of chemical ecology have become an issue of debate (Wilson, 1975; Beauchamp *et al*., 1979; Katz and Shorey, 1979; Martin, 1980; Weldon, 1980; Rutowski, 1981; Smith, 1981; Pasteels, 1982; Alves, 1988). Despite this, the nomenclature described above has become widely accepted, and although alternative terminology has been suggested (*e.g.* Chernin, 1970; Martin, 1980), it is seldom used (Alves, 1988).

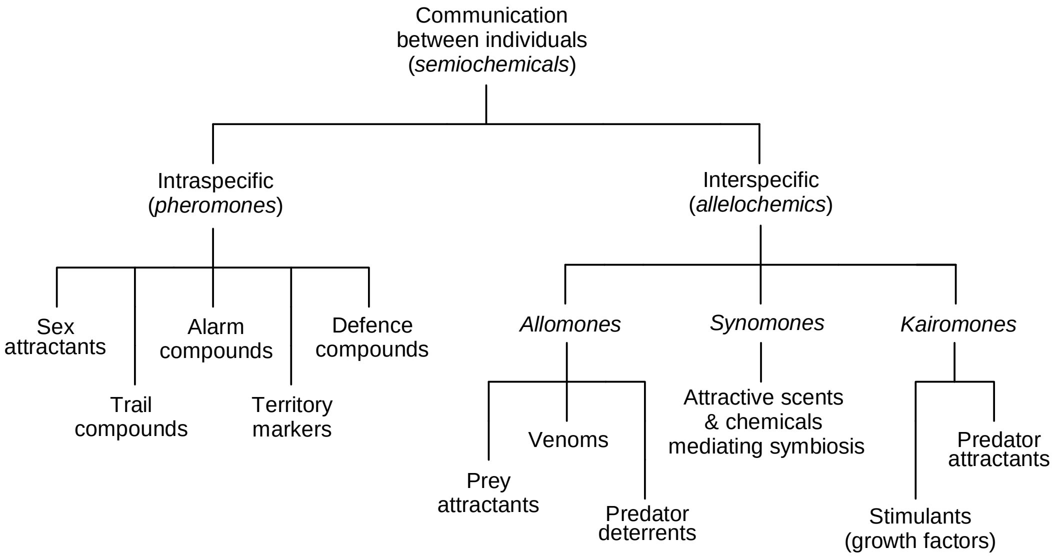


Figure 1.1. Summary of the chemical interactions which have an important influence on ecological relationships (adapted from Naylor, 1984).

In addition to uncovering the remarkably subtle and intricate ways in which organisms communicate *via* chemistry, terrestrial chemical ecology has also provided an insight into the intriguing nature of the semiochemicals involved. The plethora of compounds implicated in ecological interactions are structurally complex and exhibit extreme diversity and idiosyncrasy in their temporal, taxonomic and ontogenetic distribution (Haslam, 1985, 1986; Berenbaum, 1995). Their only true unifying feature is that they are natural products having no known role in the primary, protoplasmic metabolism (*i.e.* respiration, photosynthesis or other critical metabolic processes) of the organisms that produce them (Haslam, 1985, 1986; Mann, 1987). Since the exact metabolic function of many of these compounds was not apparent at the time of their discovery by chemists and biochemists, these natural products were assumed play a subsidiary role in the biology of the producing organism and have therefore been broadly termed 'secondary metabolites' (Haslam, 1985, 1986; Mann, 1987).

Secondary metabolites are generally classified by their structural characteristics and biosynthetic origin into one of four groups: phenolics (aromatic and poly-aromatic compounds), isoprenoids (terpenes and steroids), polyketides or alkaloids (Natori, 1974; Haslam, 1985, 1986; Mann, 1987; Mann *et al.*, 1994). The division between primary and secondary metabolites, however, is not always clear-cut; there are many rare and unusual amino acids, peptides and fatty acids which should be classed as secondary metabolites, while a large number of steroidal alcohols play an essential structural or hormonal role in most organisms and should therefore be considered primary metabolites (Mann, 1987). To compound this problem further, the processes of primary and secondary metabolism are intimately related (see figure 1.2) as primary metabolism provides the precursors for the various secondary metabolic pathways (Natori, 1974; Haslam, 1986; Mann, 1987; Mann *et al.*, 1994; Berenbaum, 1995).

It is now generally accepted that secondary metabolites have arisen by genetic accident (mutation and recombination) and persist as a Darwinian response to various environmental pressures (Haslam, 1985, 1986; Williams *et al.*, 1989; Berenbaum, 1995). Nevertheless, a detailed knowledge of the complex ecological role that these chemicals fulfil is required in order to evaluate the hypotheses which have been proposed to explain how and why such metabolites have evolved (Haslam, 1986; Williams *et al.*, 1989, Christophersen, 1991). This then, is the ultimate goal of chemical ecology and enormous challenges remain both in elucidating the roles of individual compounds as well as in determining their evolutionary origins (Mitchel-Olds *et al.*, 1998).

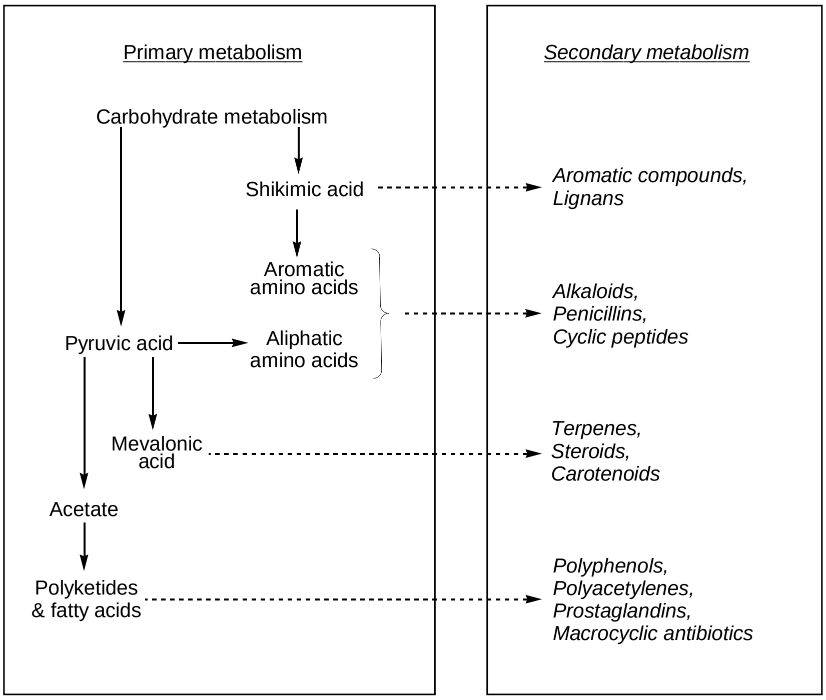


Figure 1.2. The relationship between primary and secondary metabolism (adapted from Natori, 1974 and Mann, 1987).

1.2. Marine chemical ecology: origins and development

In contrast to the situation pertaining to the vast knowledge of the natural products chemistry and chemical ecology of terrestrial systems, comparable studies of marine systems have only been undertaken relatively recently (Scheuer, 1977; Fenical, 1982; Paul, 1992a). When SCUBA became widely available in the 1960s, so did the first opportunity for scientists to observe at first-hand the biologically diverse and ecologically complex marine environment (Fenical, 1982; Hay and Fenical, 1996). Subsequent chemical investigations have produced a fundamental knowledge of marine secondary metabolism (Fenical, 1982) and to date several thousands of secondary metabolites have been isolated from marine organisms (Faulkner, 2000 and previous reviews in this series). Many of these natural products differ markedly from terrestrial secondary metabolites: a large proportion are halogenated and often possess novel chemical structures and functional groups that are unprecedented among terrestrial organisms (Fenical, 1982; Paul, 1988; Hay and Fenical, 1996; Hay, 1996). This is hardly surprising when considered from a phylogenetic perspective: many marine plants and invertebrates represent almost exclusively marine phyla and will have evolved secondary metabolic processes quite distinct from their terrestrial counterparts (Fenical, 1982). More recently, chemical studies of marine organisms have been directed toward defining the biological functions of these remarkable metabolites (Scheuer, 1977; Fenical, 1982; Van Alstyne and Paul, 1988; Pawlik, 1993; Hay, 1996).

Initially, advances in the field of marine chemical ecology were largely made by performing ecological tests on secondary metabolites which had already been isolated and characterised because of their interesting or unusual chemical properties (Hay, 1996). These initial studies relied heavily on the concepts and theories developed through terrestrial chemical ecology and were often experimentally based upon terrestrial investigations (Paul, 1992a). Early investigations focused on pheromones and defensive allomones of marine invertebrates (notably crustaceans, cnidarians, holothurians, and echinoderms: reviewed by Scheuer, 1977; Naylor, 1984), plant-herbivore interactions (Hay and Fenical, 1988; Hay, 1992; Paul, 1992b; Steinberg, 1992) and the chemical ecology of shell-less marine molluscs and their prey (Schulte and Scheuer, 1982; Faulkner and Ghiselin, 1983; Faulkner, 1988; Faulkner, 1992).

More recently, with concerted collaboration between organic chemists and marine ecologists, and improvements in experimental design and chromatographic techniques, many of the compounds isolated from marine species have been shown to serve as efficient defences against predators, competitors, fouling organisms and pathogens, play roles in reproductive processes, and act as cues for settlement or metamorphosis of larvae in tropical, temperate and Antarctic benthic communities (Naylor, 1984; Bakus *et al.*, 1986; Coll and Sammarco, 1988; Coll *et al.*, 1990; Paul, 1992a; Pawlik, 1992, 1993; Lambert and Todd, 1994; McClintock, 1994; Hay, 1996; Hay and Fenical, 1996; Morse and Morse, 1996; McClintock and Baker, 1997, 1998). Additionally, recent studies are also disclosing the possible importance of such chemical interactions in pelagic marine communities (McClintock and Janssen, 1990; Shaw *et al.*, 1995a; 1995b; Hay, 1996).

Despite the extensive progress made in the field of marine chemical ecology, there is much still to be accomplished: Pawlik (1993) states that there remains a large body of biological literature that implicates the action of semiochemicals in marine ecological phenomena. Many of the metabolites responsible for these interactions remain unknown as they are frequently present in trace amounts, are not conspicuous in spectroscopic screening of crude organism extracts or may be inactive in routine bioactivity assays (Hay, 1996). To address these limitations, more emphasis has been placed on the design of ecologically relevant bioassay techniques (comprehensively reviewed by Hay *et al*., 1998). Accordingly, marine chemical ecology has matured into a research area in which chromatographic and spectroscopic investigations are performed in tandem with ecologically relevant assay methods to reveal the underlying molecular basis of many of the inter- and intra-specific interactions observed in the marine environment (for recent reviews see: Bakus *et al*. 1986; Hay and Fenical, 1988, 1996; Duffy and Hay, 1990; Paul, 1992a; Pawlik, 1992, 1993; Fenical, 1993; McClintock, 1994; Avila, 1995; Hay, 1996; McClintock and Baker, 1997, 1998).

However, Hay *et al.* (1998) believe that, although a great deal can still be accomplished within the traditional confines of marine chemical ecology, the most significant future advances in the field will be achieved by better integrating chemical ecology with physiology, neurobiology, molecular biology and population, community and ecosystem ecology. The development of methodologies to allow this integration will be extremely challenging, but if this is successfully accomplished it will afford a much deeper understanding of the mechanisms affecting biological change in marine ecosystems and yield fundamental insights into the ecology and evolution of marine and aquatic communities.

This thesis documents an investigation into the chemical ecology of a bryozoan-whelk symbiosis, which was prompted by observations made during an ecological study of a predator-prey role reversal in a benthic ecosystem (Barkai and McQuaid, 1988).

Chapter 2

The study organisms and their interaction: *Jasus lalandii, Burnupena papyracea* and *Alcyonidium nodosum*

The rock lobster genus *Jasus* (Parker) has a wide distribution in the southern hemisphere (Barnard, 1950; Heydorn, 1969a, 1969b) comprising seven extant species at separate southern circumpolar localities (George and Main, 1967; Pollock, 1995). The majority of *Jasus* species occupy shallow-water, cool-temperate environments (mainly between latitudes 27°-45° south), although the genus does include one subtropical species endemic to the east coast of Australia and northern New Zealand (George and Main, 1967; Pollock, 1995). The southern African species, *Jasus lalandii* (Milne Edwards), is an important benthic predator associated with the kelp-beds on the west coast of South Africa (Heydorn, 1969a; Newman and Pollock, 1974; Field *et al.*, 1977; Griffiths and Seiderer, 1980; Barkai and Branch, 1988a, 1988b, 1988c; Barkai and McQuaid, 1988). Although it has a recorded distribution extending from north of Walvis bay in Namibia to the Gonubie River mouth, near East London in South Africa (Barnard, 1950; Heydorn 1969a), it is only found at high densities on the South African west coast between Cape Point (34° 30' S) and Easter Cliffs (25° 20' S) and forms the basis of a commercially important fishery in this area (Pollock, 1979; Pollock and Beyers, 1981; Pollock *et al.*, 1982).

Jasus has been shown to be an opportunistic predator and scavenger (Barkai and Branch, 1988a), its diet consisting mainly of mussels, sea urchins, sponges, algae, barnacles, small molluscs and crustaceans (Heydorn, 1969a; Newman and Pollock, 1974; Pollock, 1979; Barkai and Branch, 1988a; Barkai, Davis and Tugwell, 1996). The species is extremely voracious, substantially modifying subtidal communities (Branch *et al.* 1987, Barkai and Branch, 1988b) to the point that, when present in large numbers, it may eliminate most of its benthic prey (Barkai and Branch, 1988c). The extent to which *Jasus lalandii* may effect the benthic ecology of the South African west coast became apparent from the work of Barkai (1987).

A study of the benthos of two near-shore islands, Marcus Island (17° 58' E, 33° 02' S) and Malgas Island (17° 55' E 33° 03' S) in the Saldanha Bay area showed that, although they lie only a few kilometres apart, have similar bottom topography and experience comparable physical conditions, their subtidal benthic communities differ markedly (Barkai, 1987; Barkai and Branch, 1988a;

Barkai and McQuaid, 1988). Benthic communities at Marcus Island are dominated by extensive beds of the black mussel, *Choromytilus meridionalis*, as well as large populations of holothurians, sea urchins and whelks. In contrast, Malgas Island is dominated by a large population of *Jasus lalandii* together with a dense seaweed flora. Although the two communities appear completely different, it has been suggested that they represent alternative states of the same ecosystem, and that the contrast between the two communities is the consequence of the presence or absence of intense predation by rock lobsters (Barkai, 1987; Branch *et al.*, 1987; Barkai and Branch, 1988a; Barkai and McQuaid, 1988).

Predation pressure on adult *Jasus lalandii* is limited on the west coast (Heydorn, 1969a; Pollock and Beyers, 1981): post-larval stages are preyed upon to a limited degree by the Cape fur seal, octopuses (which are rare), hagfish, various species of dogsharks, and the bony fish Red Roman and Mussel-cracker. Therefore the near absence of *Jasus* around Marcus Island is intriguing. An explanation proposed by Barkai and McQuaid (1988) is that, in the past, a combination of factors, such as pollution from fish factories (Newman and Pollock, 1973; Christie and Moldan, 1977), oxygen-deficient water (Newman and Pollock, 1973; Pollock and Beyers, 1981) and construction work on the iron ore jetty in the bay (Beckley, 1981), depleted the population of rock lobsters in this area. In the absence of their principal predator, whelk densities at Marcus increased to the extent that they now inhibit *Jasus* from recolonising this area. The normal predator-prey interaction between *Jasus* and the whelks has been reversed at Marcus Island, and whelks have been observed to overwhelm and consume experimentally introduced rock lobsters (Barkai and McQuaid, 1988).

In a detailed study of this predator-prey role reversal, Barkai and McQuaid (1988) also revealed that two whelk species, *Argobuccinum pustulosum* (Lightfoot) and *Burnupena papyracea* (Bruguière), are immune to predation by *Jasus* and can be found in areas where rock lobster densities are high. In a series of laboratory feeding trials, they concluded that *A. pustulosum* is afforded physical protection from predation by virtue of its thick shell. *B. papyracea*, however, is defended through a symbiotic relationship with a commensal bryozoan, *Alcyonidium nodosum*

(O'Donoghue and de Watteville), which covers the shell of the whelk. It was suggested that this protection has a chemical basis (Barkai and McQuaid, 1988).

The ctenostomate bryozoan genus *Alcyonidium* (Lamouroux) is completely marine (Thorpe and Ryland, 1979) and extremely speciose (Thorpe *et al.*, 1978; Peck *et al.*, 1995). Most species inhabit shallow coastal waters as epiphytes of inshore algae or epizoites of molluscs, crustaceans, bryozoans or hydroids, although a few species develop large, erect, fleshy colonies attached by narrow stalked bases to hard substrata (Peck *et al.* 1995). A single species, *Alcyonidium disciforme* (Smitt), lives unattached on the surface of soft substrata (Kvitek, 1989). Colony form in *Alcyonidium* species is usually simple, developed as an encrusting, unilaminar sheet of monomorphic autozooids: erect colonies form by surface budding of new autozooids, building up a multilaminar structure, only the outer layer of which bears functioning polypides (Peck *et al.*, 1995). *Alcyonidium nodosum* is an encrusting species which lives exclusively on *Burnupena papyracea*, totally covering the shell and creating a nodular, orange or purple cloak over the whelk (O'Donoghue and de Watteville, 1944; O'Donoghue, 1956). When unencrusted, *B. papyracea* shells possess a papery outer layer (Pers. Obs.; Branch *et al.*, 1994) which may facilitate settlement and attachment of *Alcyonidium* and explain the specificity of the bryozoan-whelk relationship.

The presence of *Alcyonidium nodosum* on *Burnupena papyracea* shells could reduce predation on its host whelk in several ways. Bryozoans have yielded a large number of biologically active natural products consisting mainly of alkaloids or macrocycles, although some terpenes and simple organic molecules have also been isolated (Christophersen, 1985; Anthoni *et al.*, 1990; Faulkner, 2000 and previous reviews in this series). The only species of *Alcyonidium* which has been examined chemically, *A. gelatinosum*, contained a potent biologically active component, the (2-hydroxyethyl)dimethylsulfoxonium ion (Carlé and Christophersen, 1980 and 1982), which has been implicated in causing an eczematous, allergic, contact dermatitis in North Sea fishermen (Fraser and Lyell, 1963; Turk *et al.*, 1966; Carlé and Christophersen, 1980; 1982). It is therefore reasonable to assume that the defence conferred on *B. papyracea* by *A. nodosum* may have a chemical basis. Alternatively, some physical factor may also be responsible for the defence of the whelk as predation on bryozoans may trigger the rapid production of defensive spines or chitinous structures (Harvell, 1984; 1986; 1990; 1992). Induction of this type of physical defence in *A. nodosum* as a response to damage caused by *Jasus* whilst feeding on *B. papyracea* could result in reduced consumption of the host whelk.

The presence of *Alcyonidium nodosum* on *Burnupena papyracea* shells could also protect the whelk if bryozoan encrustation significantly increased shell strength. Given a choice between different species of mussels, *Jasus lalandii* shows a distinct preference for species with weaker shells (Griffiths and Seiderer, 1980). The skeletal material of some encrusting bryozoans has been calculated to be of comparable strength to coral, echinoid spine and bivalve shell (Best and Winston, 1984). Though a feature of the genus *Alcyonidium* is a lack of calcification of the exoskeleton (Thorpe and Ryland, 1979), *A. nodosum* nevertheless forms a firm layer over its host's shell (O'Donoghue and de Watteville, 1944). It is therefore possible that the presence of bryozoans on *B. papyracea* may increase the strength of the whelk's shell. Finally, it is also possible that, when covered with a layer of bryozoan, the whelks are no longer conspicuous as prey items or cannot be handled efficiently by *J. lalandii*, thus decreasing the number of bryozoan encrusted individuals consumed.

The objective of this study was to determine whether *Alcyonidium nodosum* deters feeding by *Jasus lalandii* through physical or chemical means.

Chapter 3

Materials and Methods

3.1. *Jasus lalandii* feeding preference experiments

Feeding assays were run to assess the basis of the prey preference exhibited by *Jasus* (the data obtained for these assays are reproduced in tables A.1 – A.10 of the appendix). Mature *Jasus lalandii* (n = 6, mean carapace lengths ± SD = 110.1 ± 18.7 mm) were obtained from the University of Cape Town and transported to Grahamstown on ice. On arrival, the rock lobsters were immediately transferred to separately filtered sea-water aquaria held under constant environmental conditions (temperature = 12 ° C, 12 hour light/dark cycle). After 4 weeks under these conditions the rock lobsters were feeding regularly, were assumed to be laboratory acclimatised and the group was submitted to a series of four feeding assays (see sections 3.1.1, 3.1.2, 3.1.3 and 3.1.4). The duration of each bioassay was 14 days and a period of 14 days was allowed to pass between bioassays. In bioassays involving live prey, the control and test prey items made available to each rock lobster were always of similar size. While no bioassays were being performed, the rock lobsters were fed daily on a diet of mussels.

Burnupena papyracea and *B. cincta* (Röding) were collected subtidally using SCUBA from depths of between 3 and 10 m in False Bay, off Simonstown (34°14' S; 18°26' E), South Africa. The whelks were stored in sea-water aquaria until they were transported in aerated sea-water at approximately 15 ° C. On arrival in Grahamstown the whelks were maintained in re-circulating sea-water aquaria at 12 ° C under ambient light conditions and fed on a diet of mussels.

The *Alcyonidium nodosum* used in the assays was obtained by scraping the shells of *Burnupena papyracea* collected subtidally using SCUBA from depths of between 4 and 9 m in Saldanha Bay on the west coast of South Africa (33° 02' S; 17° 58' E). The bryozoan was both transported and stored frozen. Portions of *A. nodosum* were lyophilised as required with a yield of approximately 0.50 g freeze-dried *Alcyonidium* per whelk.

A further portion of *Alcyonidium nodosum* (1396 g wet weight) obtained from 519 whelks was freeze-dried (dry weight 259.50 g) and extracted into a 1:1 dichloromethane/methanol mixture under nitrogen at -20 °C for 24 hours in the dark. Following filtration, the red/orange organic extract was concentrated *in vaccuo* to yield a dark red oil (7.76 g; 15 mg per whelk) which was stored at -20 °C in the dark.

3.1.1. Bioassay 1: encrusted versus scraped Burnupena papyracea

Each rock lobster was offered three *Burnupena papyracea* encrusted with *Alcyonidium nodosum* and three *B. papyracea* from which the commensal bryozoan had been removed by scraping. Consumed whelks were replaced daily. This bioassay is essentially a replicate of one of the tests performed by Barkai and McQuaid (1988).

3.1.2. Bioassay 2: pellets containing Alcyonidium nodosum versus control food pellets

A method for suspending freeze-dried *Alcyonidium nodosum* in a gelatinous matrix was modified from the techniques described by Chanas and Pawlik (1995) and Pawlik *et al*. (1995). Pellets were prepared daily as follows. Sodium alginate (1.00 g), freeze-dried, powdered mussel (0.50 g) and freeze-dried, powdered *A. nodosum* (2.00 g) were added to deionised water (30 ml) and the mixture stirred vigorously until homogenous. After centrifuging to remove air bubbles, the resulting paste was loaded into a syringe and extruded into calcium chloride solution (250 ml, 0.25 M). As *Jasus* feeds by tearing off and ingesting small pieces of its prey, the sodium alginate paste was left in the $CaCl_2$ solution until it was hard throughout. After 90 minutes, the resultant gel was removed, blotted dry and cut into pellets (radius = 0.35 cm, length = 2.00 cm; each containing approximately 50 mg freeze-dried *A. nodosum*). Pellets were attached to separate, marked mussel shell valves using Bostic® super glue and the glue allowed to dry thoroughly before the shells were presented to *Jasus lalandii*. Control pellets were prepared in the same manner using sodium alginate and freeze-dried mussel only. Each rock lobster was offered three fresh test pellets and three fresh control pellets daily.

3.1.3. Bioassay 3: encrusted Burnupena papyracea versus B. cincta

Each rock lobster was offered three *Burnupena papyracea* encrusted with *Alcyonidium nodosum* and three specimens of *B. cincta*, a species which has unencrusted shells. Consumed whelks were replaced daily.

3.1.4. Bioassay 4: Burnupena papyracea encrusted with live Alcyonidium nodosum versus B. papyracea encrusted with dead A. nodosum

Each rock lobster was offered three *Burnupena papyracea* encrusted with untreated *Alcyonidium nodosum* and three *B. papyracea* encrusted with bryozoans which had been treated with liquid nitrogen (the control prey item) immediately prior to use. Liquid nitrogen was applied to the bryozoans using a cotton wool pad. The bryozoans were thoroughly exposed but care was taken not to allow any liquid nitrogen to come into contact with the whelk. Treated shells did not differ visibly from controls and were marked to allow recognition during the bioassay. Consumed whelks were replaced daily and treated whelks replaced after a few days if not consumed in that period.

3.1.5. Replicate assays: bioassays 5 - 9

Bioassays 1-4 were repeated using a second group of *Jasus lalandii* (n = 7) which were obtained and maintained as before. The mean size of this group (mean carapace length ± SD = 121.3 ± 19.6 mm) was not significantly different from the first group of *Jasus* (two sample *t*-test [two-tailed], 11 d.f., $t = -1.050$, $P = 0.316$). These assays were run in a random sequence (5 = 4, 6 = 3, 7 = 1 and 8 = 2). A third group of smaller *Jasus* (n = 12, mean carapace length ± SD = 87.2 ± 7.0 mm, two sample *t*-test [two-tailed], 23 d.f., $t = -4.428$, $P < 0.001$) was used to run a third replicate (assay 9) of bioassay 2 (pellets containing freeze-dried bryozoan versus control pellets).

Separate batches of *Alcyonidium nodosum* were used for the preparation of pellets in assays 8 and 9.

3.1.6. Bioassay 10: pellets containing Alcyonidium extract versus control food pellets

The third group of *Jasus* (n = 12) was also used to test the *Alcyonidium* extract. The method used for suspending freeze-dried *Alcyonidium* described in section 3.1.2 was modified for use with the *Alcyonidium* extract as follows. Sodium alginate (2.00 g) was suspended in an ether solution (10.0 ml) of the *Alcyonidium* dichloromethane/methanol extract (0.54 g). The solvent was removed by rotary evaporation to give a yellow powder. Freeze-dried mussel (1.00 g) and deionised water (60.0 ml) were added to the treated alginate and the mixture stirred vigorously until homogenous. The resultant paste was then prepared for presentation to *Jasus* as described in section 3.1.2 to yield 36 treated pellets each containing approximately 15 mg of *Alcyonidium* extract (i.e. an amount comparable with that contained within a colony of bryozoans on a single *Burnupena papyracea*). Control pellets were prepared in the same manner using sodium alginate, ether and freeze-dried mussel only. Each rock lobster was offered three fresh test pellets and three fresh control pellets daily.

3.2. *Artemia salina* (Leach) larvicidal bioassays of *Alcyonidium nodosum* extracts

The crude dichloromethane-methanol extract of *Alcyonidium nodosum* was subjected to further fractionation by solvent partition. A portion of the extract (126 mg) was dissolved in methanol (5.0 ml) and partitioned between hexane (2 × 10.0 ml) and 10% aqueous methanol (5.0 ml). The two fractions were separated and the hexane fraction dried over anhydrous magnesium sulphate and concentrated *in vaccuo* to yield a hexane-soluble fraction as an orange oil (44 mg). The aqueous methanol fraction was dried azeotropically *in vaccuo* to give a methanol-soluble fraction as a red oil (79 mg). The toxicities of the dichloromethane-methanol crude organic extract (COE), the hexane-soluble extract (HSE) and the methanol-soluble extract (MSE) were evaluated in a standard larvicidal assay widely employed in the isolation of bioactive natural products (Meyer *et al.*, 1982; Solis *et al.*, 1992).

3.2.1. Hatching of Artemia salina cysts

Brine shrimp cysts (Rainbow Aquarium and Pet Supplies, Port Elizabeth) were hatched by incubation in filtered sea-water at 26 °C under constant aeration in the light for 48 hours. Hatched instar II and III larvae were separated from cyst shells and unhatched cysts by phototaxis and used immediately.

3.2.2. The Artemia bioassay

The three *Alcyonidium* extracts (5 mg each) were dissolved in ethanol (500 µl), and 100 µl of the resulting solutions were added to filtered sea-water (650 µl) to give bioassay stock solutions (1.0 mg extract/750 µl). Control stock solutions were prepared using ethanol (100 µl) and sea-water (650 µl) only. Stock solutions (150 µl) were put into separate wells of a 96-microwell plate. Serial dilutions were made in the wells so that addition of *Artemia salina* nauplii in 100 µl of sea-water would yield test solutions of concentration 400, 200, 100 and 50 µg/ml. Each concentration was replicated six times for each extract. Control wells were included in each experiment, being prepared in the same way using the control stock solution. A sea-water suspension of *A. salina* nauplii containing about 20 larvae (100 µl) was added to each well by autopipette and the plates covered and incubated at 26 °C for 24 hours. Plates were then examined under a binocular microscope (×20 magnification) and the numbers of dead (non-motile) nauplii in each well counted. Methanol (100 µl) was then added to each well and after 10 minutes the total numbers of shrimp in each well recorded and the percentage mortality of *Artemia* in each well calculated.

The numbers of *Artemia* responding to the treatment after 24 hours, total number exposed and percentage mortality are shown in table A.11 of the appendix.

3.3. Spectroscopic screening of the *Alcyonidium nodosum* extracts

Each *Alcyonidium* extract prepared in section 3.2 (crude organic extract, hexane soluble extract and methanol soluble extract) was screened by ^{1}H nuclear magnetic resonance (NMR) spectroscopy at 400 MHz on a Bruker Avance 400 spectrometer in deuterochloroform, deuterium oxide, deuteromethanol and deuterobenzene. Four deuterated solvents of diverse polarity were chosen to insure that diverse chemical compounds with varying solubilities could be detected by NMR spectroscopy. The extracts (5 mg) were dissolved in deuterated solvent (1.0 ml) and filtered through a small plug of cotton wool to remove precipitates. The ^{1}H NMR spectra (16 scans, 2 dummy scans, spectral width 8012.8 Hz; 20.0 ppm) were recorded at 300.0 K on a 5mm broad band multinuclear probe with sample spinning using a 90° radio frequency pulse of 13.50 μseconds duration and 2.0 dB power level, a dwell time of 62.40 μseconds and a relaxation delay of 1.00 second. Spectra were calibrated to residual protonated solvent ($CDCl_3$, 7.250 ppm; D_2O, 4.820 ppm; CD_3OD, 3.300 ppm; C_6D_6, 7.150 ppm).

For comparative purposes, the ^{1}H NMR spectrum of the (2-hydroxyethyl)dimethylsulfoxonium ion isolated from *Alcyonidium gelatinosum* by Carlé and Christophersen (1980) was simulated using NMRSimul Version 1.0 (Dipartemento di Chimica delle Sostanze Naturali, Napoli).

3.4. Analysis for volatile chemicals produced by *Alcyonidium nodosum*

Evidence that bryozoans can produce volatile chemicals (Christophersen and Carlé, 1978; Blackman *et al.*, 1992) prompted an analysis for volatile components of *Alcyonidium nodosum*. This was accomplished by solid phase microextraction (SPME) of the headspace above *A. nodosum* samples onto a fibre coated with polydimethylsiloxane (100 μm film thickness).

Subsequent desorption, separation and examination of volatile components was performed by gas chromatography on a Hewlett Packard HP 6890 GC system fitted with a 50% phenyl-50% methylsiloxane DB17 column (30 m × 0.25 mm I.D., stationary phase thickness 0.25 µm, J & W Scientific, California) and a flame ionisation detector (FID).

A typical procedure for the extraction and analysis was as follows: the SPME fibre and DB17 column were both conditioned for one hour before each set of experiments (front inlet temperature and oven temperature: 250 ° C). Two control extractions testing the extraction vial/septum and sea-water from the aquaria in which *Alcyonidium nodosum* were being maintained were performed and analysed prior to extractions of *Alcyonidium*. Live bryozoans from two *Burnupena papyracea* were removed from the whelks by carefully scraping the shells and immediately placed in a glass vial fitted with a silicone septum. The vial was placed in a water bath (approximately 60 ° C) and the headspace extracted for 15 minutes. The extractives were loaded onto the column by thermal desorption from the SPME fibre in the front inlet of the GC (temperature: 250 °C; splitless injection mode; fibre exposed for 5 minutes) and eluted using the following oven parameters: 120 °C for 10 min, 10 °C/min to 250 °C, held at 250 °C for 5 min. The bryozoan extraction was performed three times (different colonies sampled each time) in every set of experiments and the full set of five experiments (two independent controls and three replicated tests) repeated three times.

3.5. Scanning electron microscopy of *Alcyonidium nodosum*

The possibility of an inducible defence mechanism being employed by the bryozoan colonies enshrouding the *Burnupena papyracea* whelks was investigated as follows. The surfaces of *Alcyonidium nodosum* colonies on whelks (n = 6) which had been exposed to contact with *Jasus lalandii* (sharing the same aquaria for one week) were compared with the surfaces of bryozoan colonies on untreated whelks (n = 6) using scanning electron microscopy. Samples of bryozoan colonies were removed from each shell and fixed over-night in 5% glutaraldehyde in filtered sea-water at 0 ° C. Once fixed, the bryozoan tissue was dehydrated through a graded ethanol series and infiltrated with amyl acetate by treatment with a graded ethanol/amyl acetate series to facilitate preparation by critical point drying. Samples were then sputter-coated with gold and observed with a Jeol JSM 840 scanning electron microscope.

3.6. Comparison of shell strengths of encrusted *Burnupena papyracea*, scraped *B. papyracea* and *B. cincta*

The compressional force required to the crack shells of encrusted *Burnupena papyracea*, scraped *B. papyracea* and *B. cincta* was determined using an Instron 4301 materials testing system. Shells were evaluated with a 5000 Newton, internally calibrated load cell. Force was applied continuously and without shock at a rate of 100 mm per minute. When the instrument detected shell cracking, force was removed and the force required to cause shell failure recorded.

Before testing, each whelk was placed on its aperture and positioned so that force was applied at the highest point of the first whorl of the shell. Force was applied to the shells through a steel rod (3.4 mm diameter). The total number tested, minimum, and maximum shell length for individuals of the three groups tested were: encrusted *Burnupena papyracea* (n = 68, 17.4 - 62.9 mm); scraped *B. papyracea* (n = 68, 16.3 - 62.9 mm); and *B. cincta* (n = 45, 20.0 - 48.3 mm). There was no significant difference in the mean size of whelks among groups (ANOVA, 2 d.f., F-ratio = 1.430, P = 0.242).

3.7. Statistical analyses

Statistical analyses were performed using Statistica for Windows 1999 edition (StatSoft Inc., Tulsa, Oklahoma) and Statgraphics version 5.0 (STSC Inc., Rockville, Maryland). Before analysis, all data were tested for normality using the Kolmogorov-Smirnov goodness-of-fit test (Sokal and Rohlf, 1981) and homogeneity of variances was assessed with Cochran's C test (Underwood, 1981).

3.7.1. Jasus lalandii feeding assays

The bioassay data were analysed by two methods: repeated measures ANOVA and χ^2 goodness-of-fit. The repeated measures ANOVAs were performed on untransformed data for daily consumption of prey items by individual rock lobsters. Data were not normally distributed (Kolmogorov-Smirnov test, $P < 0.01$ for all bioassays) and variances were not homogenous for assays 3, 4, 6 and 7 (Cochran's test, $P < 0.05$). Transformation of the data (arcsine, logarithmic and square-root) did not significantly reduce heteroscedasticity or deviations from normality, and, in the absence of a non-parametric equivalent to repeated measures ANOVA, we were obliged to rely on the robustness of ANOVA to yield meaningful results (Underwood, 1981; Zar, 1999).

Jasus is an extremely voracious feeder, and animals of the size we used consume an average of about 10 mussels per day in the laboratory (Griffiths and Seiderer, 1980). In these experiments we limited the amount of food available to well below this level, and so assumed that feeding of rock lobsters on each prey type could be regarded as independent (see Peterson and Renauld, 1989). Therefore, the repeated measures model had one within-subjects factor, "day", and two between-subjects factors, "prey type" and "lobster". In certain assays there were insufficient degrees of freedom to allow use of the preferred multivariate repeated measures analysis described by O'Brien and Kaiser (1985). Consequently, both the between-subjects and within-subjects factors were analysed by univariate methods using the Greenhouse-Geisser adjustment to the degrees of freedom to correct for violations of the assumption of sphericity among the repeated measurements (O'Brien and Kaiser, 1985; Prince, 1995).

Yates' corrected χ^2 goodness-of-fit tests (Sokal and Rohlf, 1981; Zar, 1999) were performed on the cumulative, pooled totals of control and test prey items consumed by the end of each assay.

3.7.2. Artemia salina larvicidal assays

Artemia salina mortality in both the controls and treatments were converted to proportions, the data arcsine transformed (Zar, 1999) and analysed by two-way ANOVA with treatment and concentration as factors.

3.7.3. Shell strengths

The relationship between shell length and force required to cause shell failure was investigated for each whelk type by model II regression analysis (using linear, exponential, multiplicative and exponential models), the slopes of the resulting correlations being tested for significance by ANOVA (Zar, 1999). Significant differences in the means of shell strength data between the three whelk types were analysed by ANOVA followed by Tukey's honest significant difference test.

Chapter 4

Results

4.1. *Jasus lalandii* feeding assays

The results of the *Jasus* assays are shown in figures 4.1, 4.2 and 4.3 as cumulative, pooled totals of prey items consumed against day as this best illustrates preferences in prey selection. The results of statistical analyses are reported in table 4.1 and the analysis results concerned with the effect of prey type on feeding of *Jasus* summarised in table 4.2 to show contrasts between the outcomes of replicated tests. Comparison of the *Jasus* assay data indicates that there is a large degree of inconsistency between replicated tests; none of the assays gave an unequivocal result (*i.e*. $P < 0.05$ in both statistical methods for all replicates of a particular assay) and replicates of assays often gave different results with respect to feeding preference (see table 4.2).

On examination of the results of the repeated measures ANOVA, it becomes apparent that there was a high degree of variability in the behaviour of *Jasus* during the assays. In the majority of assays (assays 2, 3, 5, 7, 8, 9 and 10), individual lobsters ate at different rates so that “lobster” had a significant effect (ANOVA, $P < 0.05$). Also in assays 1, 2, 4, 8, 9 and 10, “day” had a significant effect (ANOVA, $P < 0.01$) indicating that the number of prey items eaten per day was not constant during these experiments.

In an attempt to compensate for behavioural effects of the test animal, the *Jasus* assay data were analysed by two statistical methods; ANOVA and χ^2 goodness of fit. Repeated measures ANOVA, although it is the more powerful and stringent method, is sensitive to variability in the consumption of prey items (especially control items) between rock lobsters. In all assays it was often common for some rock lobsters to eat all control prey items whilst others ate none: this situation yields a large spread of data about the mean number of control prey items consumed. Such a scenario may then result in ANOVA reporting a non-significant effect of prey type on feeding of rock lobsters even if a preference for control items was present in the assay. Despite this limitation the use of ANOVA methods in the analysis of the *Jasus* feeding assay data is desirable as, in addition to furnishing information regarding preference of prey type, it also gives some measure of the differences of feeding behaviour amongst lobsters as well as the daily variation in the consumption of prey items.

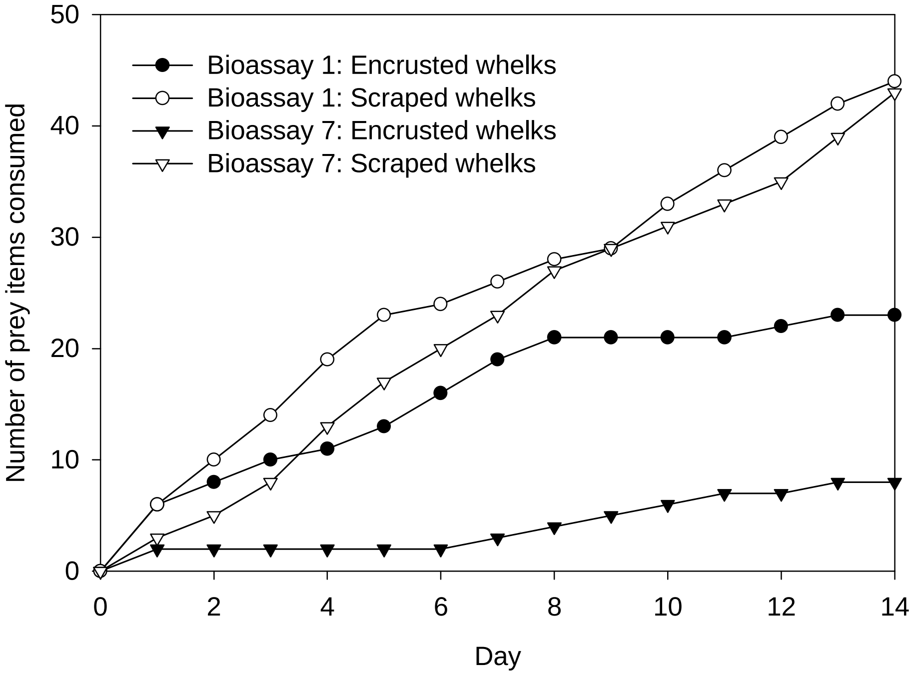
Recognising the potential problems inherent in the application of an ANOVA model in this situation, *Jasus* assay data were also analysed by the χ^2 goodness-of-fit test. Although it may be considered less conservative than the repeated measures ANOVA, χ^2 goodness-of-fit is not subject to the same limitations with regard to behavioural variability of test animals. This method utilises the cumulative totals of prey items consumed during the assay to test for a preference of prey type by comparison of the observed numbers of prey items consumed with the values expected if no preference was displayed. These two individual statistical tests should therefore be considered complimentary rather than disparate, and in general, analysis of data by χ^2 gave the same result as analysis by ANOVA (the exceptions being assays 1 and 4).

One instance in which statistical tests yielded different results for the same assay was in the first experiment, testing for a preference between encrusted and scraped *Burnupena papyracea* (assay 1, figure 4.1A). Analysis by ANOVA indicated a non-significant effect of prey type, but a significant effect was displayed in the χ^2 test. When this assay was repeated on a second group of *Jasus* (assay 7, figure 4.1A), a clear preference for unencrusted *B. papyracea* was observed (ANOVA and χ^2, $P < 0.05$).

Inconsistent results were obtained in assays testing the effect of freeze dried *Alcyonidium* in food pellets (assays 2, 8 and 9, figure 4.1B). Assays 8 and 9 gave clear evidence of a preference for control pellets (ANOVA and χ^2, $P < 0.001$ for both assays) although no preference was displayed in assay 2, where a much lower overall feeding rate was also observed. The result of assay 2 therefore seems anomalous and should be treated with caution as it may have been affected by some discrepancy in the handling or preparation of the *Alcyonidium* used in this assay.

In a situation analogous to that observed for assays 2, 8, and 9, separate groups of *Jasus* gave contrasting results when given a choice between encrusted *Burnupena papyracea* and *B. cincta*. The first replicate of this assay (assay 3, figure 4.2A) indicated a clear preference for *B. cincta* (ANOVA and χ^2, $P < 0.001$) whilst no preference was observed in the second replicate (assay 6, figure 4.2A, ANOVA and χ^2, $P > 0.05$).

A



B

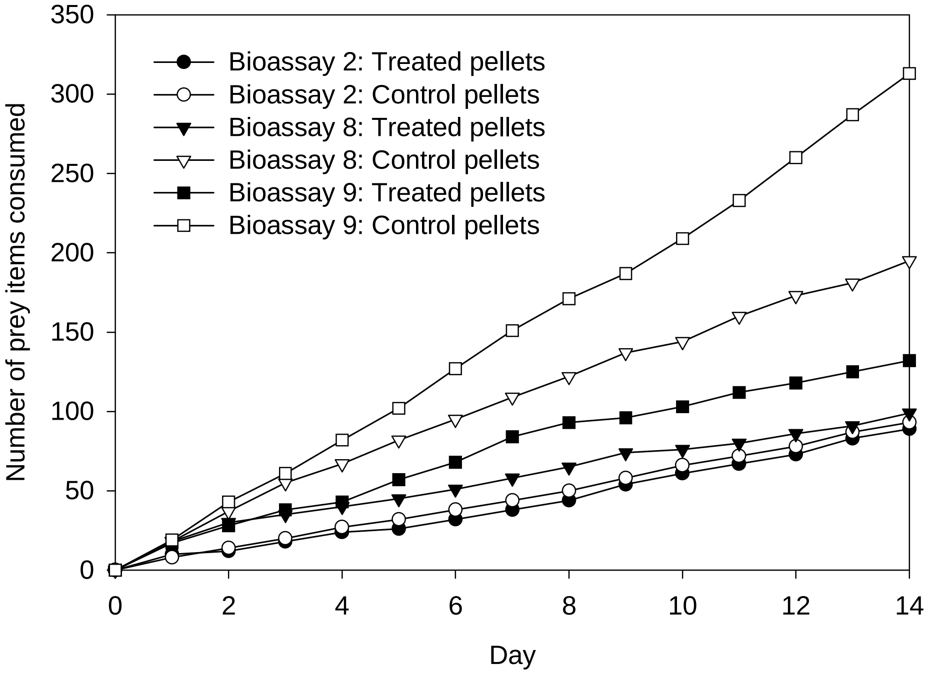
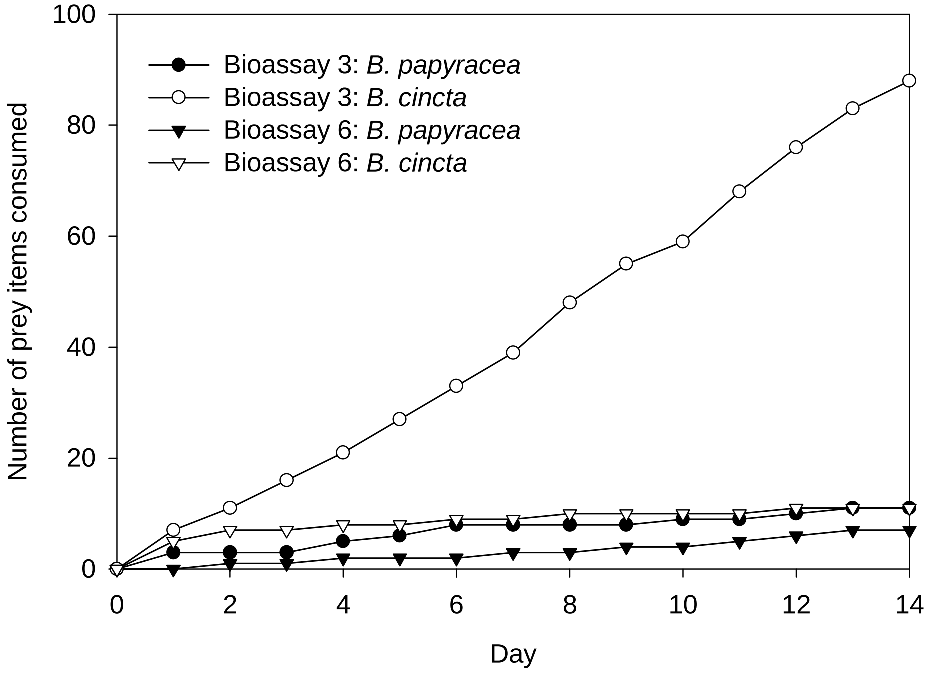


Figure 4.1. Cumulative, pooled totals of food items taken by *Jasus lalandii* in (A) assays 1 (n = 6) and 7 (n = 7): encrusted versus scraped *Burnupena papyracea*; and (B) asssays 2 (n = 6), 8 (n = 7) and 9 (n = 12): pellets containing freeze-dried *Alcyonidium* versus control pellets.

A



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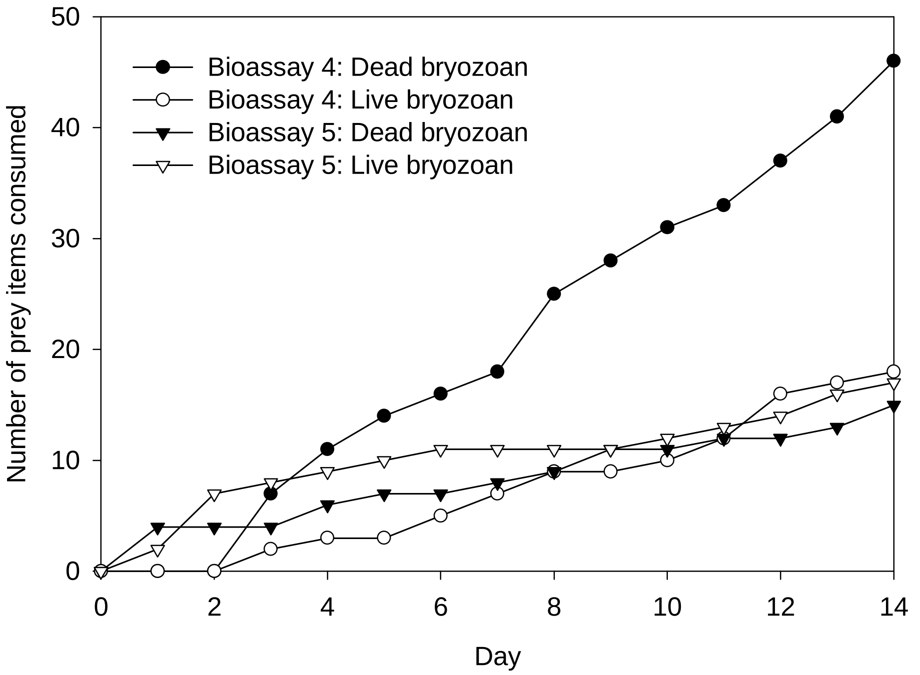

Figure 4.2. Cumulative, pooled totals of food items taken by *Jasus lalandii* in (A) assays 3 (n = 6) and 6 (n = 7): *Burnupena papyracea* versus *B. cincta*; and (B) asssays 4 (n = 6) and 5 (n = 7): Liquid nitrogen treated versus untreated *B. papyracea*.

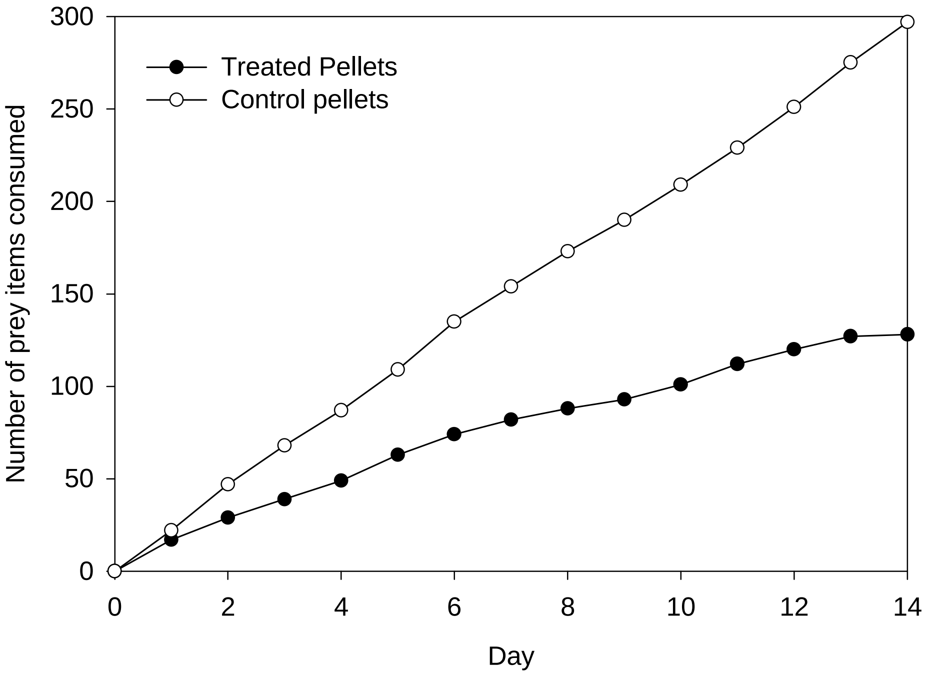

Figure 4.3. Cumulative, pooled totals of food items taken by *Jasus lalandii* in assay 10 (n = 12): pellets containing *Alcyonidium* extract versus control pellets.

Experiments testing the deterrent effect of live *Alcyonidium* versus dead (liquid nitrogen treated) *Alcyonidium* also gave problematic results: assay 4 (figure 4.2B) gave a non-significant result by ANOVA (although this assay gave a relatively high F-ratio; 3.004, corresponding to $P = 0.096$) but a significant result when analysed by χ^2 goodness-of-fit. The replicate of this test (assay 5, figure 4.2B) indicated no preference for control prey items by either statistical method.

The final bioassay (assay 10, figure 4.3), which tested the effect of the *Alcyonidium nodosum* crude organic extract on prey selection of *Jasus*, gave a clear result with respect to feeding preference. Both ANOVA and χ^2 methods indicated a distinct deterrent effect for the pellets containing *Alcyonidium* extract ($P < 0.001$ in both analyses).

To recapitulate then, in bioassays 1, 3, 4, 7, 8, 9 and 10 there was a significant feeding preference for control prey items indicated by the χ^2 goodness-of-fit test ($P < 0.05$) but assays 1 and 4 showed no significant effect of prey type when analysed by repeated measures ANOVA ($P > 0.05$). In the remaining assays (2, 5 and 6), no feeding preference was indicated by either test (ANOVA and χ^2, $P > 0.05$). The result obtained for assay 2 (pellets containing freeze-dried *Alcyonidium* versus control pellets) seems to be anomalous; the two replicates of this assay (assays 8 and 9) involved significantly higher consumption of prey items and indicate that *Jasus* exhibits a marked preference for control food pellets (figure 4.1B).

During the assays showing no effect of food type with live prey items (assays 5 and 6), five of the seven *Jasus* moulted and consumption of prey items was consequently extremely low. This effect was constant throughout the two assays (no significant difference observed for “day” in repeated measures ANOVA, $P > 0.05$) and indicates that rock lobsters were feeding poorly during these periods. While not ignoring the outcome of these assays (although it would not be unreasonable to exclude these results *a priori* because the test animals were in a physiologically stressed state), more confidence is placed in the results of the respective replicates (assays 4 and 3) of these assays.

Therefore, the results of the rock lobster feeding preference experiments indicate that, in bioassays showing significant results, *Jasus* preferred scraped *Burnupena papyracea* to encrusted (assay 7, figure 4.1A); *B. cincta* to encrusted *B. papyracea* (assay 3, figure 4.2A); *B. papyracea* treated with liquid nitrogen to untreated (assay 4, figure 4.2B); and control pellets to pellets containing either *Alcyonidium nodosum* (assays 8 and 9, figure 4.1B) or an *Alcyonidium* extract (assay 10, figure 4.3). In summary, *Jasus* avoided live *Alcyonidium* when given a choice and likewise avoided food pellets containing *Alcyonidium* or its chemical extract.

Table 4.1. Results of repeated measures ANOVAs and χ^2 goodness-of-fit tests on *Jasus lalandii* feeding preference data. ANOVAs performed on data for daily consumption. χ^2 tests performed on cumulative, pooled totals on termination of the assays.

			ANOVA†		Yates' corrected χ^2	
Bioassay	Factor	d.f.	F-ratio	Significance	χ_c^2	Significance
1. Encrusted versus scraped *B. papyracea*	Prey Type	1	1.309	P = 0.264	6.597	P = 0.011
	Lobster	5	2.588	P = 0.052		
	Day	13	3.331	P = 0.003		
2. *Alcyonidium* treated pellets versus control pellets	Prey Type	1	0.552	P = 0.465	0.093	P = 0.761
	Lobster	5	266.621	P << 0.001		
	Day	13	10.584	P << 0.001		
3. Encrusted *B. papyracea* versus *B. cincta*	Prey Type	1	28.643	P << 0.001	59.899	P << 0.001
	Lobster	5	6.223	P < 0.001		
	Day	13	1.855	P = 0.092		
4. Liquid N_2 treated versus untreated *B. papyracea*	Prey Type	1	3.004	P = 0.096	12.266	P < 0.001
	Lobster	5	1.441	P = 0.246		
	Day	13	3.341	P = 0.004		
5. Liquid N_2 treated versus untreated *B. papyracea*	Prey Type	1	0.336	P = 0.856	0.156	P = 0.697
	Lobster	6	2.860	P = 0.027		
	Day	13	1.878	P = 0.088		
6. Encrusted *B. papyracea* versus *B. cincta*	Prey Type	1	0.211	P = 0.650	0.944	P = 0.344
	Lobster	6	1.039	P = 0.421		
	Day	13	2.351	P = 0.074		
7. Encrusted versus scraped *B. papyracea*	Prey Type	1	5.594	P = 0.025	24.039	P << 0.001
	Lobster	6	3.321	P = 0.014		
	Day	13	0.930	P = 0.493		
8. *Alcyonidium* treated pellets versus control pellets	Prey Type	1	31.241	P << 0.001	31.350	P << 0.001
	Lobster	6	7.348	P << 0.001		
	Day	13	14.024	P << 0.001		
9. *Alcyonidium* treated pellets versus control pellets	Prey Type	1	83.361	P << 0.001	73.622	P << 0.001
	Lobster	11	12.777	P << 0.001		
	Day	13	6.497	P << 0.001		
10. *Alcyonidium* extract treated pellets versus control pellets	Prey Type	1	48.082	P << 0.001	67.205	P << 0.001
	Lobster	11	10.870	P << 0.001		
	Day	13	5.652	P << 0.001		

†Interactions have been excluded for brevity.

Table 4.2. Summary of results of repeated measures ANOVAs and χ^2 goodness-of-fit tests for effect of prey type on *Jasus lalandii* feeding preference. Bioassays are grouped by prey type.

Bioassay	Assay No.	No. of *Jasus*	Analysis results	
			ANOVA	χ^2
Encrusted versus scraped *B. papyracea*	1	6	N.S.*	P < 0.05
	7	7	P < 0.05	P << 0.001
Encrusted *B. papyracea* versus *B. cincta*	3	6	P << 0.001	P << 0.001
	6†	7	N.S.	N.S.
Liquid nitrogen treated versus untreated *B. papyracea*	4	6	N.S.	P < 0.001
	5†	7	N.S.	N.S.
Alcyonidium treated pellets versus control pellets	2‡	6	N.S.	N.S.
	8	7	P << 0.001	P << 0.001
	9	12	P << 0.001	P << 0.001
Alcyonidium extract treated pellets versus control pellets	10	12	P << 0.001	P < 0.001

*N.S. = No significance, P > 0.05. †Assays in which more than one *Jasus* moulted. ‡Assay involving low overall feeding by *Jasus*.

4.2. *Artemia salina* toxicity assays

The results of the *Artemia salina* assays are shown in figure 4.4 as mean percentage *A. salina* mortality against extract concentration. The result of the two-way ANOVA is summarised in table 4.3. All three of the *Alcyonidium nodosum* extracts failed to show significant biological activity against *A. salina* larvae at the concentrations tested (ANOVA, all effects non-significant at P > 0.05; less than 15% mortality in all wells). The mean percentage of mortality (± SD) for each of the treatments in the assay (pooled concentrations) were: organic crude extract, 2.2 ± 4.2%; hexane soluble extract, 0.6 ± 1.7%; methanol soluble extract, 3.6 ± 3.6%; and control, 2.0 ± 3.4%.

The mean number of *A. salina* larvae (± SD) exposed to each treatment in this assay was 19.3 ± 4.6 (n = 96). It should therefore be noted that the death of only a few larvae in one treatment could give rise to relatively large (> 0.05) value of the mortality proportion for that treatment. This situation would account for the seemingly large variability exhibited in the *A. salina* mortality data shown in figure 4.4.

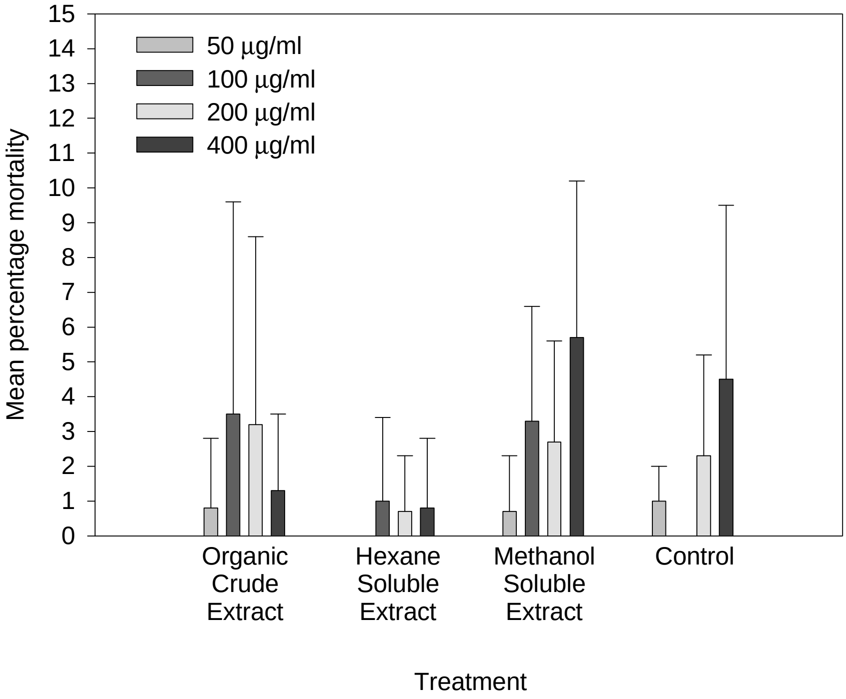

Figure 4.4. Relationship between mean mortality and treatment in *Artemia salina* assays (n = 6 for each concentration, error bars show standard deviation).

Table 4.3. ANOVA table for *Artemia salina* assay data.

Factor	d.f.	F-ratio	Significance
Treatment (controls and extracts)	3	2.421	P = 0.072
Concentration	3	2.230	P = 0.091
Interaction (Treatment-Concentration)	9	0.755	P = 0.658

4.3. Spectroscopic screening

The simulated ^1H NMR spectrum of the (2-hydroxyethyl)dimethylsulfoxonium ion isolated from *Alcyonidium gelatinosum* is shown in figure 4.5. The ^1H spectrum of this compound contains two distinct proton singlets at 4.30 ppm and 3.90 ppm integrating in the ratio of 2:3 (Carlé and Christophersen, 1980). The ^1H NMR spectra obtained for the *Alcyonidium nodosum* extracts are shown in figures 4.6, 4.7 and 4.8. No resonances similar to those exhibited by the (2-hydroxyethyl)dimethylsulfoxonium ion were observed in any of the NMR spectra.

The presence of common types of biologically active natural products including unsaturated terpenes, acetogenins, alkaloids, phenolics or peptides would be indicated in the spectra of the *Alcyonidium* extracts by protons resonating between approximately 3 and 10 ppm. No deshielded signals corresponding to these types of secondary metabolites were apparent in the spectra; only those signals consistent with the presence of hydrocarbons, long-chain fatty acids and alcohols, sterols and sugars were observed.

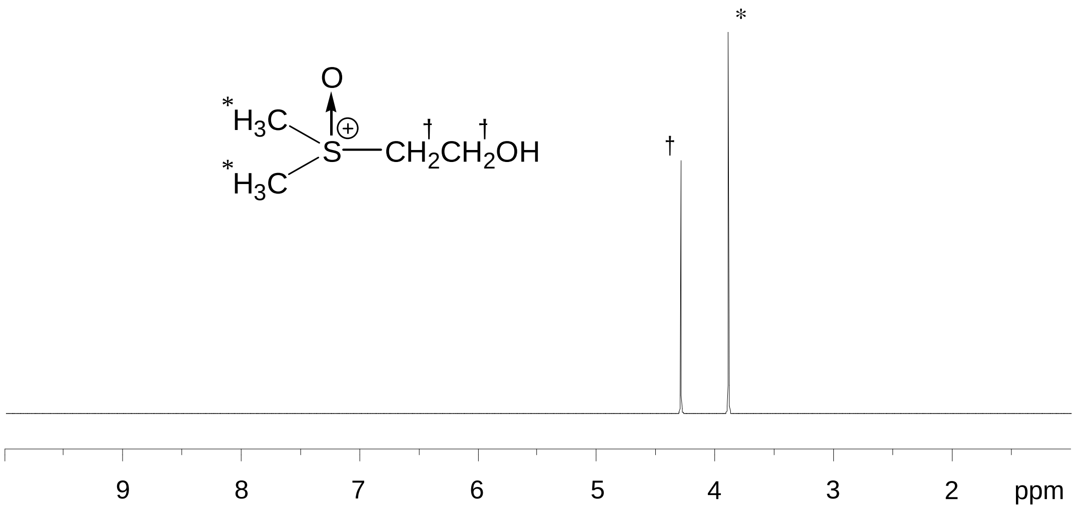

Figure 4.5. Simulated ^1H NMR spectrum of the (2-hydroxyethyl)dimethylsulfoxonium ion from *Alcyonidium gelatinosum* (in D_2O, protonated solvent resonance not shown).

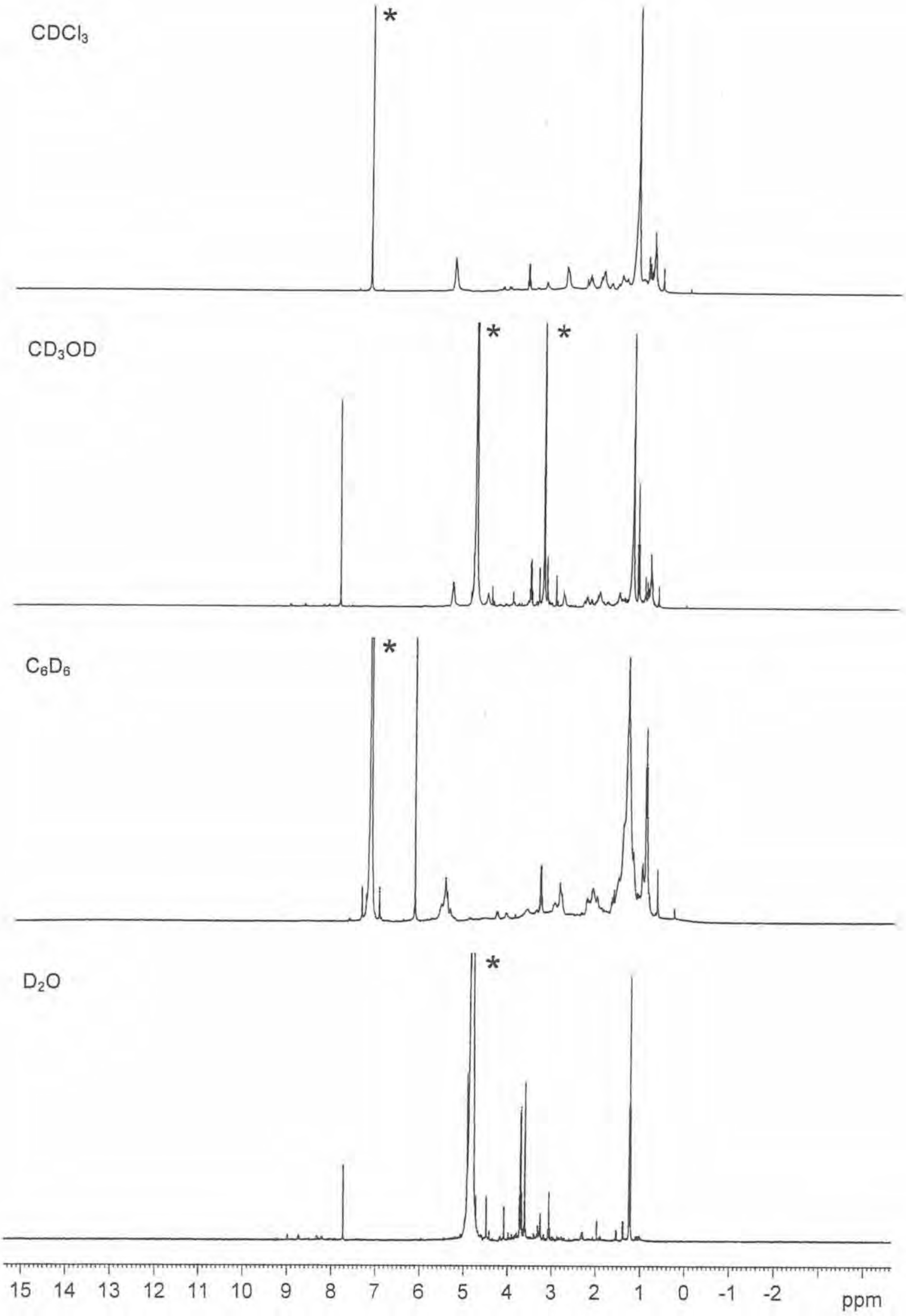

Figure 4.6. ^{1}H NMR spectra of the *Alcyonidium nodosum* crude organic extract (resonances marked by an asterisk correspond to protonated solvent)

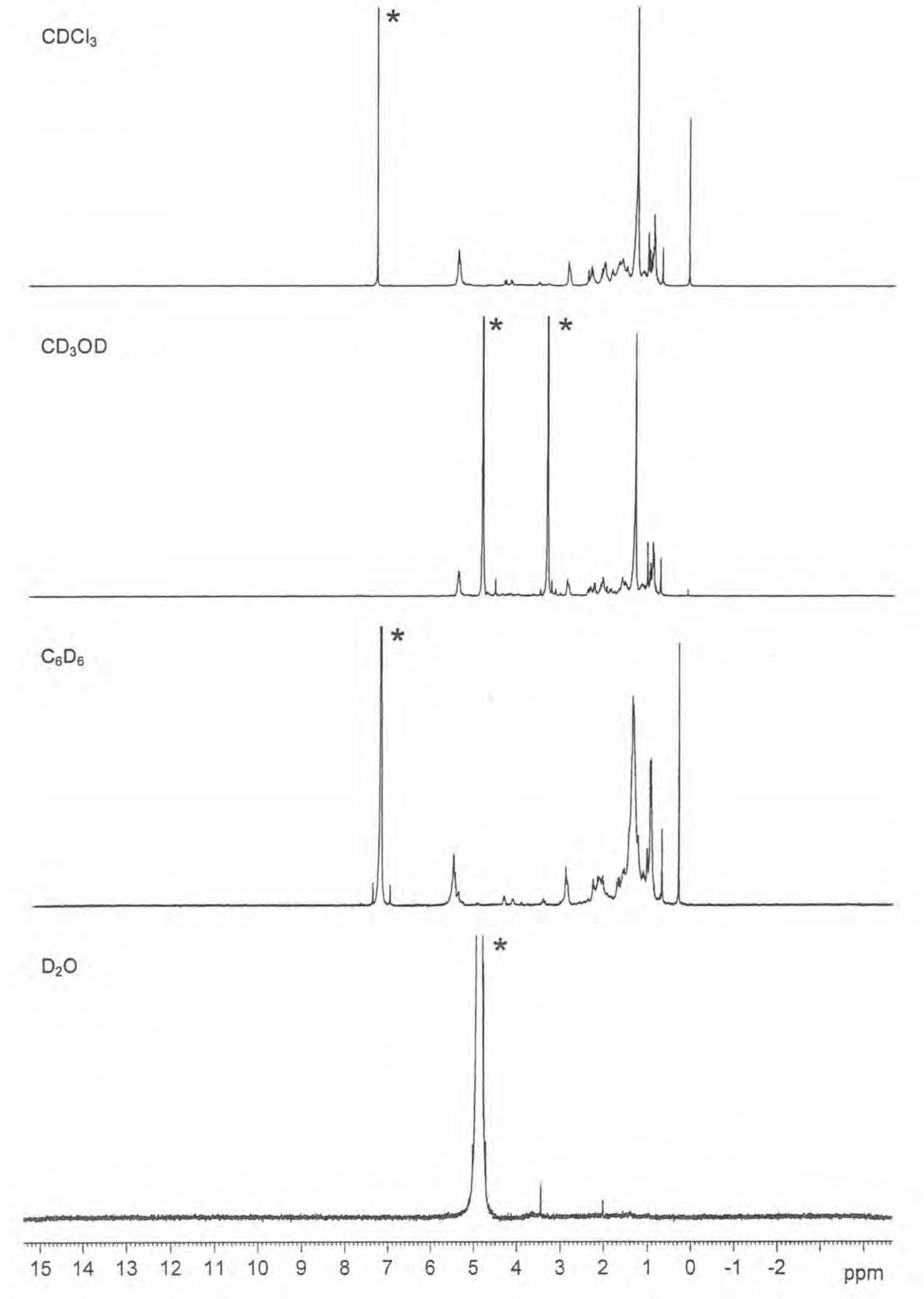

Figure 4.7. ^{1}H NMR spectra of the hexane soluble fraction of the *Alcyonidium nodosum* crude organic extract (resonances marked by an asterisk correspond to protonated solvent)

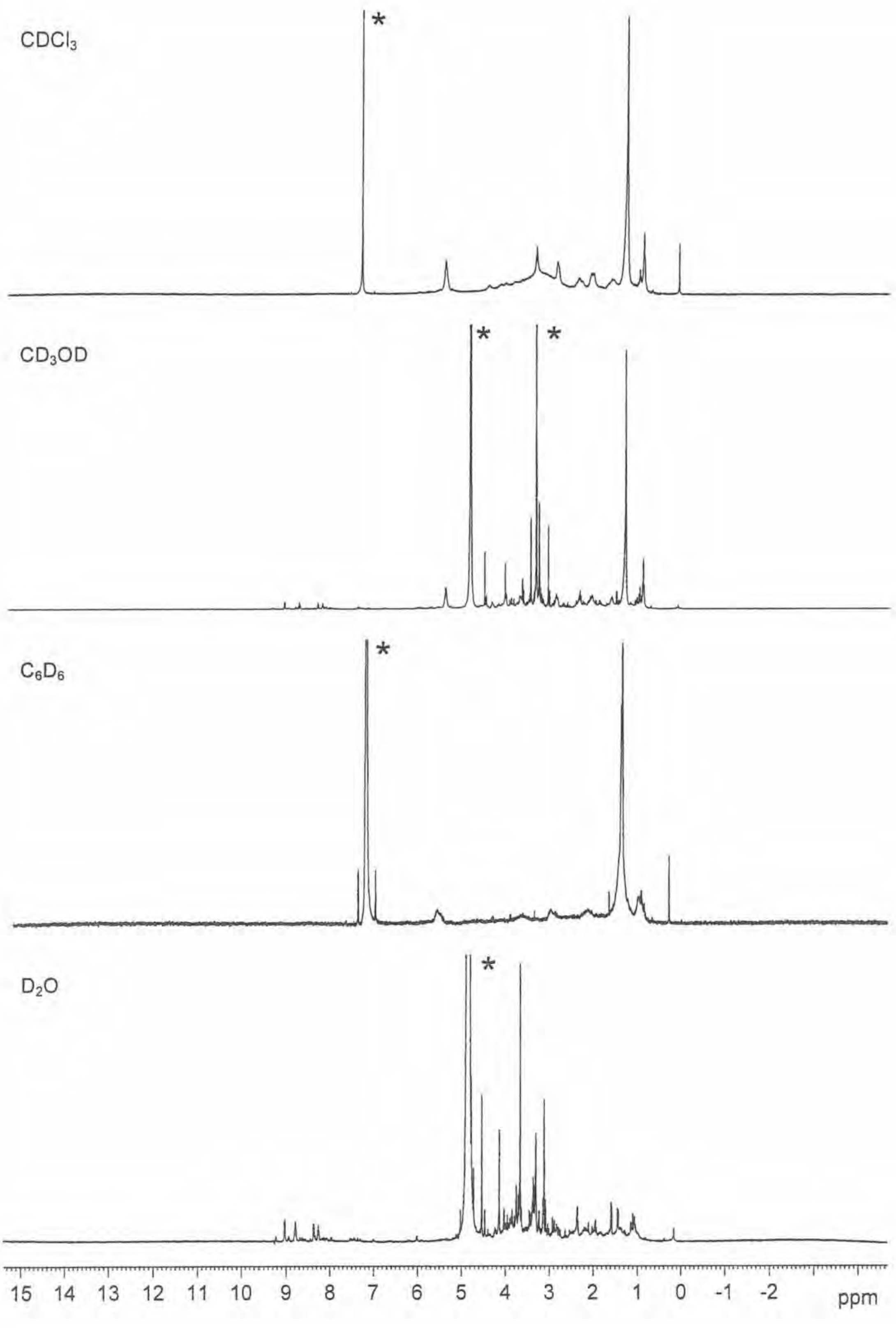

Figure 4.8. ^{1}H NMR spectra of the methanol soluble fraction of the *Alcyonidium nodosum* crude organic extract (resonances marked by an asterisk correspond to protonated solvent)

4.4. Analysis for volatile chemicals

In five out of the nine SPME bryozoan extractions, components were observed to elute from the column with retention times of between 15 and 20 minutes. These components did not elute in a regular pattern and elicited only very small responses from the FID detector (< 1 pA in all cases). The chromatograms of the remaining four bryozoan extractions did not differ from those obtained for the sea-water control extractions. Because the presence and composition of volatiles appeared to be highly variable between colonies of *Alcyonidium nodosum* and only extremely small amounts could be extracted when they were present, further characterisation of the volatile components by gas chromatography-mass spectrometry was not attempted.

4.5. Inducible defences in the bryozoan

In the experiments testing for inducible physical defence in *Alcyonidium* there were no obvious differences in the surface structure of bryozoans between replicates, therefore only typical examples of electron micrographs obtained from *Alcyonidium nodosum* colonies exposed to *Jasus lalandii* and unexposed colonies are shown in figure 4.9A and 4.9B respectively. The surfaces of the exposed bryozoans all appeared smooth and undulating with no obvious spines, spicules or other calcareous/chitinous structures. There was no difference in the surface appearance of bryozoans from whelks which had been exposed to *J. lalandii* compared to those from untreated whelks. For comparison, an example of a scanning electron micrograph showing defensive spines in the bryozoan *Membranipora membranacea* developed as a response to predation by the nudibranch *Doridella steinbergae* is reproduced from the work of Harvell (1986) and shown in figure 4.9C.

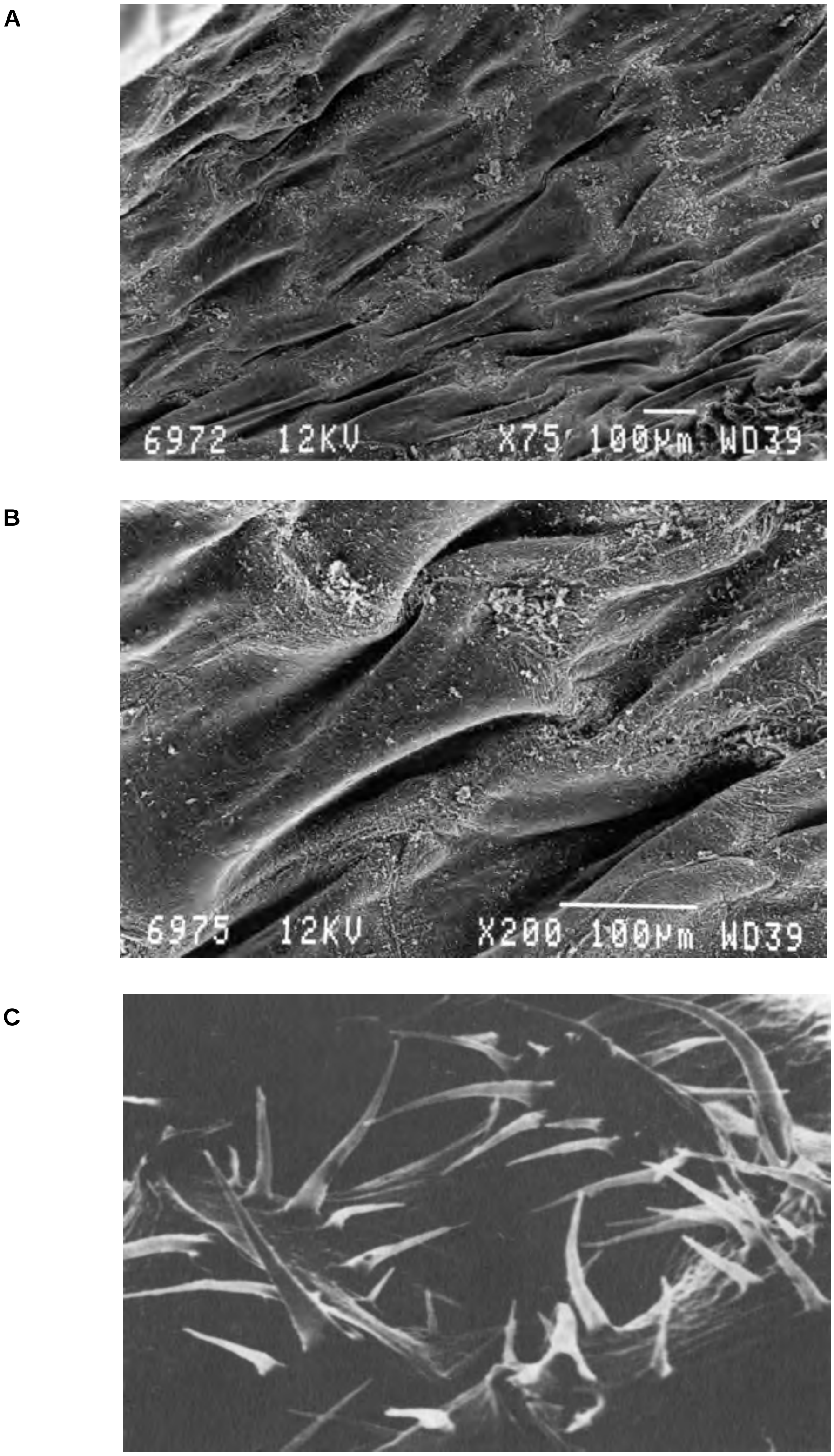

Figure 4.9. Scanning electron micrographs of (A) untreated *Alcyonidium nodosum*, (B) *A. nodosum* exposed to *Jasus lalandii*, and (C) defensive spines of *Membranipora membranacea* (field height 100µm).

4.6. Shell strengths

The results of the shell strength experiments are shown in figures 4.10, 4.11 and 4.12. The data obtained showed that, for all shell types, there was little relationship between shell length and shell strength (regression analysis, $r^2 < 0.05$ for all models; see table 4.4) and that the slopes of the resulting regressions did not differ significantly from zero (ANOVA, $P > 0.05$ for all models; see table 4.4). Therefore, the force required to crack shells could be considered independent of shell length for all three groups.

The force required to fracture the shells differed significantly for each whelk type (ANOVA, 2 d.f., F-ratio = 19.987, $P \ll 0.001$ followed by Tukey's HSD). Mean failure load was ranked as *Burnupena cincta* > encrusted *B. papyracea* > scraped *B. papyracea*. A comparison of the mean shell strength (± SE) for each whelk type is shown in Figure 4.13.

Table 4.4. Summary of regression analyses for correlation between shell length and force required for shell failure in encrusted *Burnupena papyracea*, scraped *B. papyracea* and *B. cincta*.

	Regression		Significance of regression slope (ANOVA)		
Whelk type	Model	r^2	d.f.	F-ratio	Significance
Encrusted *B. papyracea*	Linear	0.037	1	2.540	P = 0.116
	Multiplicative	0.027	1	1.842	P = 0.179
	Exponential	0.032	1	2.209	P = 0.142
	Reciprocal	0.024	1	1.625	P = 0.207
Scraped *B. papyracea*	Linear	< 0.001	1	0.025	P = 0.875
	Multiplicative	0.004	1	0.233	P = 0.631
	Exponential	0.001	1	0.054	P = 0.818
	Reciprocal	0.003	1	0.221	P = 0.640
B. cincta	Linear	0.049	1	2.210	P = 0.145
	Multiplicative	0.043	1	1.950	P = 0.170
	Exponential	0.033	1	1.479	P = 0.231
	Reciprocal	0.013	1	0.583	P = 0.449

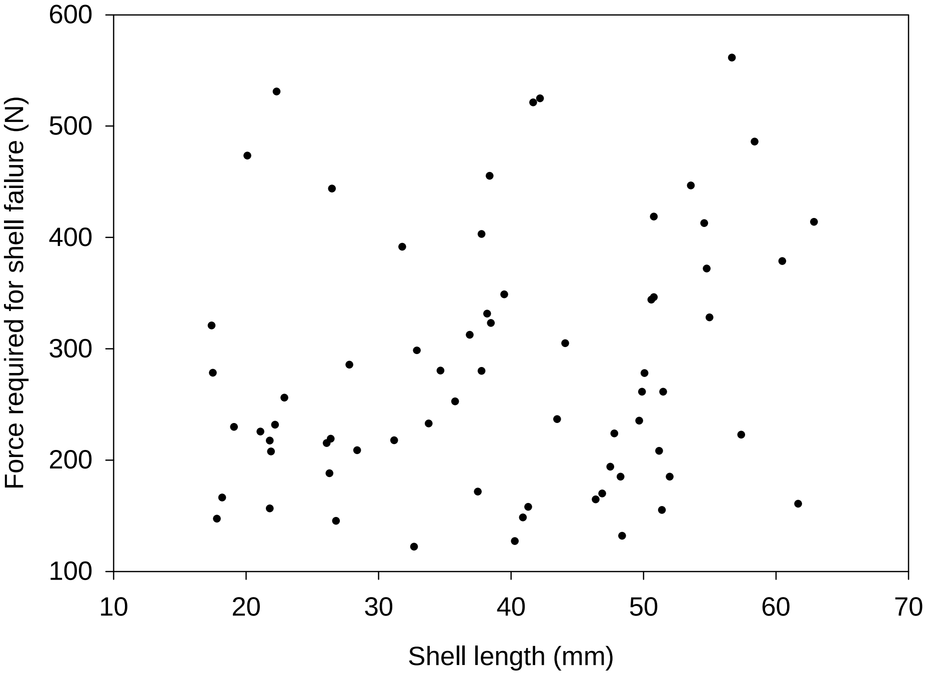

Figure 4.10. Force required to cause failure of *Alcyonidium nodosum* encrusted *Burnupena papyracea* shells (n = 68) against shell length.

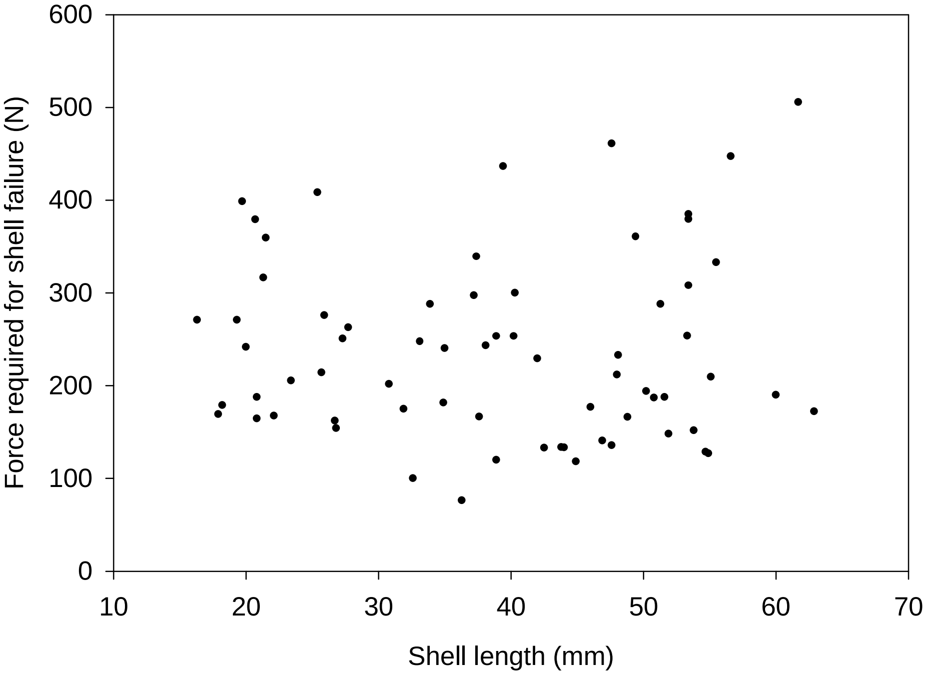

Figure 4.11. Force required to cause failure of scraped *Burnupena papyracea* shells (n = 68) against shell length.

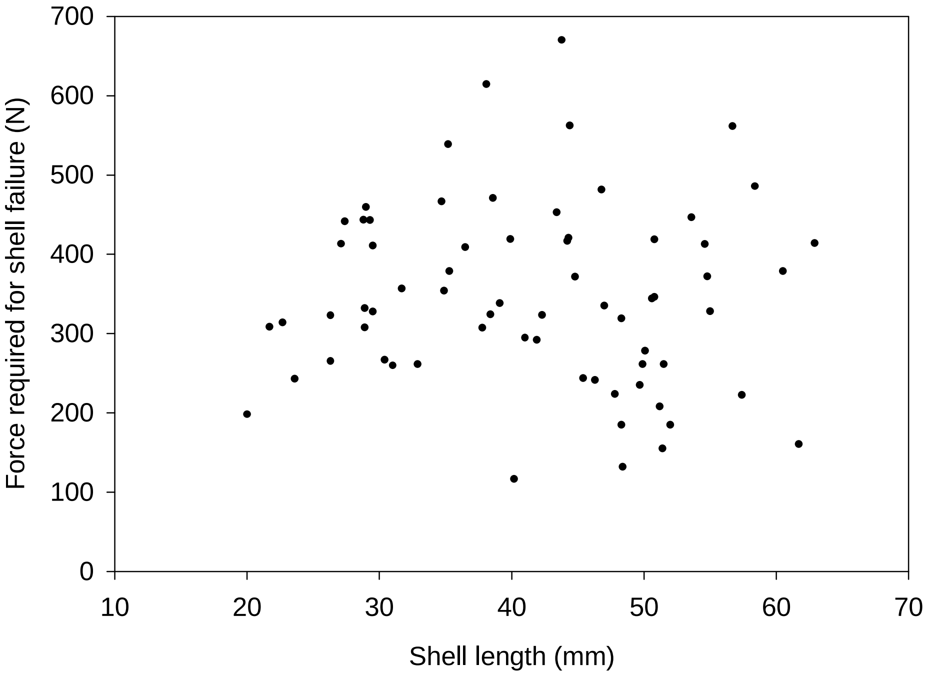

Figure 4.12. Force required to cause failure of *Burnupena cincta* shells (n = 45) against shell length.

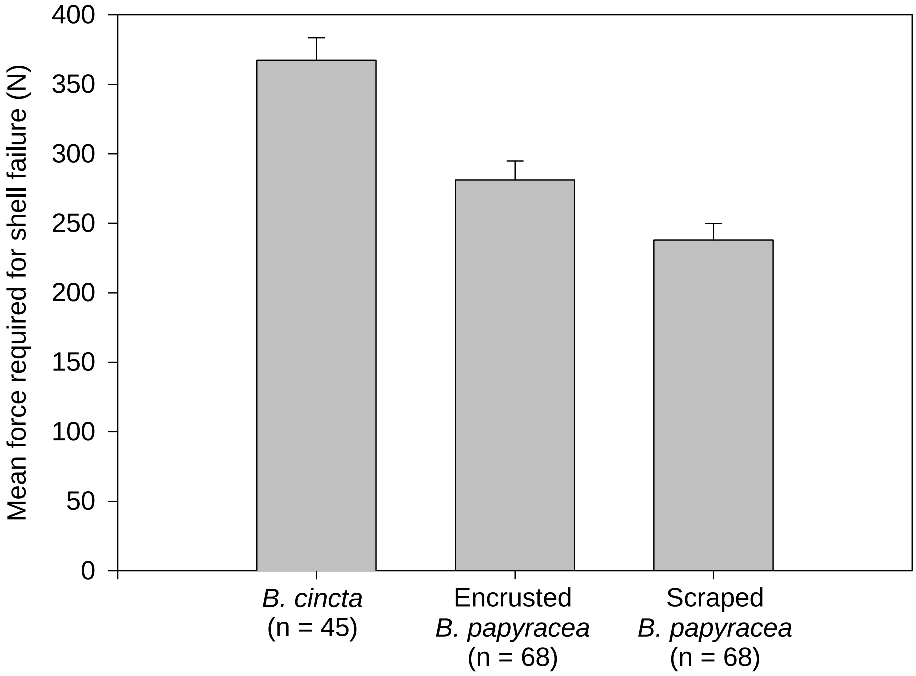

Figure 4.13. Comparison of shell strength of *Burnupena cincta*, encrusted *B. papyracea* and scraped *B. papyracea* (error bars show standard error).

Chapter 5

Discussion and Conclusions

5.1. Discussion

Over the last three decades natural products chemists have described a plethora of novel secondary metabolites from sponges, ascidians, soft corals, bryozoans, polychaetes, algae, marine microbes and other benthic and pelagic organisms which are comprehensively reviewed annually by Faulkner (2000 and previous reviews in this series). Although the natural functions of most of these secondary metabolites remain unknown, many have been demonstrated to play specific roles as pheromones or allelochemics in marine ecological interactions (Naylor, 1984; Bakus *et al.*, 1986; Coll and Sammarco, 1988; Coll *et al*., 1990; Paul, 1992a; Pawlik, 1992, 1993; Lambert and Todd, 1994; McClintock, 1994; Hay, 1996; Hay and Fenical, 1996; Morse and Morse, 1996; McClintock and Baker, 1997, 1998). The results obtained in this study indicate that the defence conferred on *Burnupena papyracea* by *Alcyonidium nodosum* is likewise chemically rather than physically based.

Although the results of repeated, independent feeding assays were often contradictory, experiments in which *Jasus* showed no preference between prey items were invariably ones in which rock lobsters were moulting and simply not feeding, or else feeding at extremely low rates. In fact, throughout this study *Jasus* proved to be remarkably behaviourally sophisticated, displaying distinct individuality. The resulting inter-replicate ‘noise’ severely affected analysis of assay results by the statistical methods commonly used in feeding preference investigations (*e.g.* the paired *t*-test or Wilcoxon’s paired sample test; see Peterson and Renaud, 1989). This problem was compounded by the fact that *Jasus* could not easily be processed in high numbers because of their large size and voracious appetite. Despite this, assays using rock lobsters gave results which are ecologically interpretable, especially when statistical methods which analyse pooled data were utilised. It could be argued that the use of the χ^2 goodness-of-fit test best describes the ecological significance of the *Jasus* feeding preference experiments as it decreases the influence that variability due to individualism of the test animals will have on the outcome of the analysis; a situation which will obviously be important when we consider the large numbers of *Jasus* responsible for the observed phenomenon in the field.

The results obtained confirm that *Jasus lalandii* shows a distinct preference for unencrusted whelks (assays 1 and 7, figure 4.1A), an observation first reported by Barkai and McQuaid (1988). Experiments investigating the basis of this feeding preference have shown that the deterrent effect of encrusted whelks is not due to physical factors resulting from the presence of the bryozoan shroud. Although certain bryozoa are known to produce defensive spines in response to predation (Harvell, 1984; 1986; 1990; 1992), *Alcyonidium nodosum* does not furnish external defensive structures on exposure to *J. lalandii*. It has also been reported that *J. lalandii* discriminates between prey items on the basis of shell strength (Griffiths and Seiderer, 1980), indeed Barkai and McQuaid (1988) suggested that the whelk *Argobuccinum pustulosum* may be protected from predation by *Jasus* by virtue of its thicker, and presumably stronger, shell. The exoskeleton of certain bryozoan species has been recorded to be of comparable strength to echinoid spine, bivalve shell or coral (Best and Winston, 1984), and the presence of *A. nodosum* was found to increase the strength of *Burnupena papyracea* shells significantly (figure 4.13). However, this also does not appear to be the factor which influences prey selection by rock lobsters. When given a choice between *B. papyracea* and *B. cincta*, *J. lalandii* preferentially fed upon *B. cincta*, even though it has a stronger shell than encrusted *B. papyracea* (assay 3, figure 4.2A).

Other physical factors which may bias prey selection by *Jasus* could be that the presence of the bryozoan alters the texture or appearance of the whelk so that rock lobsters do not recognise encrusted whelks as potential prey, or cannot efficiently manipulate whelks due to the bryozoan shroud. Whelks encrusted with *Alcyonidium nodosum* are conspicuous in the field, so that camouflage is presumably not an issue. In any event, rock lobsters tended to handle and thoroughly investigate all prey items in the assays before rejecting or accepting them (pers. obs.). Similarly, the first feeding assay comparing predation between *Burnupena papyracea* encrusted with live and dead *A. nodosum* showed that lobsters preferred whelks encrusted with dead *A. nodosum* even though the two sets of whelks were not physically or visibly different (assay 4, figure 4.2B).

The results of the first replicate of the food pellet feeding assay (no preference between pellet types, assay 2, figure 4.1B) prompted the analysis for volatile chemicals in the bryozoan which may have been lost during lyophilisation. Although not common in the marine environment, volatile chemicals have been isolated from bryozoan species (Christophersen and Carlé, 1978; Blackman *et al.*, 1992) and may well be involved in ecological processes. The technique of solid phase micro-extraction (SPME), a relatively new extraction technique that is growing in popularity for gas chromatographic analysis of pheromones (Maile *et al.*, 1998; Jones and Oldham, 1999), was employed to investigate whether *A. nodosum* produces volatile chemicals. Volatile components were extracted from *Alcyonidium nodosum* at very low levels but their occurrence in colonies was highly variable thus hindering further characterisation by gas chromatography-mass spectroscopy. This analysis, however, was subsequently made redundant by the second and third replicate of the pellet assay (assays 8 and 9, figure 4.1B) which indicated that a distinct preference for the control pellets did exist and that the results of the first replicate should be evaluated with some reservation. The anomalous result obtained for assay 2 may have been either due to some initial flaw in the technique used to prepare food pellets or simply a consequence of the idiosyncratic behaviour of the test organism. Both of these effects would have been compounded by the low *Jasus* feeding rates observed during assay 2.

It should be noted that, although the amount of *Alcyonidium* or *Alcyonidium* extract incorporated into the test pellets used in assays 2, 8, 9, and 10 seems high with respect to the amount of freeze-dried mussel, this has no bearing on the ecological relevance of these assays. *Jasus lalandii* does not have pincers and exposes whelks by chipping away the lip of the shell using its mouth-parts (pers. obs.; Barkai and McQuaid, 1988). Consequently, when attempting to feed on encrusted *Burnupena papyracea*, *Jasus* would have to endure prolonged exposure to the bryozoan before being able to access or ingest any of the whelk flesh. Therefore, these assays simply test for a deterrent effect due to the presence of *Alcyonidium* or its extract in the pellets at levels below or approximately equal to those encountered on average individual whelks in the field. The freeze-dried mussel was included only to make pellets palatable.

It would seem then that live *Alcyonidium nodosum* must contain one or more chemical components which deter feeding by *Jasus lalandii*. Indeed, both freeze-dried *Alcyonidium* and a crude organic *Alcyonidium* extract did affect prey selection in feeding assays (bioassays 8, 9 and 10, figures 4.1B and 4.3). However, spectroscopic screening of *Alcyonidium* crude extracts did not suggest the presence of structurally complex secondary metabolites such as phenolics, isoprenoids, polyketides or alkaloids that are frequently implicated in marine ecological interactions (for a comprehensive overview of the types of compounds involved see Paul, 1992a). In addition, a general bioactivity assay employing brine shrimp did not suggest the presence of biologically active metabolites in these extracts. This is rather surprising as the spectrum of bioactivity manifested as toxicity to *Artemia salina* is considered to be very broad (Meyer *et al.*, 1982; Solis *et al.*, 1993).

These findings could be a consequence of several effects. Decapod crustaceans are able to respond to nanomolar or even picomolar concentrations of certain common metabolites (Fuzessery and Childress, 1975; Schmitt and Ache, 1979). Therefore, the molecule (or molecules) deterring *Jasus* may be produced in extremely minute quantities which are below the level of detection for standard chemical and spectroscopic techniques or the *Artemia salina* assay when in a crude extract. Alternatively, the signals relating to the active chemicals may be present in the ^{1}H NMR spectra but hidden or swamped by the resonances corresponding to the large amounts of primary metabolites in the extract. Finally, the components responsible for the deterrent effect need not necessarily exhibit high broad-scale biological activity or unusual spectroscopic properties; they may simply be unremarkable, common metabolites such as steroids, fatty acid / alcohol derivatives, amino acids or peptides which are unpalatable to *J. lalandii*. Indeed, such primary metabolites have been demonstrated to deter predation in marine systems. Sterols isolated from an Antarctic soft coral deter seastar predators (Slattery *et al.*, 1997); fatty alcohol-glycerol ethers isolated from an Antarctic nudibranch and its soft coral prey have been shown to deter likely predators (McClintock *et al*., 1994); and specific amino acids, sugars, carbohydrates and glycerolipids, *etcetera*, can repel as well as attract gastropods (reviewed by Sakata, 1989).

5.2 Conclusions

In conclusion then, it has been shown that *Alcyonidium nodosum* does contain some non-volatile chemical constituent (or constituents), which deters feeding of *Jasus lalandii* and hence protects *Burnupena papyracea* from this predator. This allelochemical interaction is intriguing as it would seem that the organism which is benefiting most from the presence of the allelochemic is not the species which produces it. In this respect, the *Jasus-Burnupena-Alcyonidium* interaction described here cannot be adequately defined by the terminology currently used in chemical ecology beyond allelochemic: it is an excellent example of a complex chemically-mediated ecological interaction.

Although interactions involving three or more species are well documented in the marine environment, these usually involve specialist consumers which sequester defensive metabolites through their diet and are themselves protected from predation (*e.g.* opisthobranch molluscs feeding on algae, bryozoa, sponges or soft-corals: for reviews see Avila, 1995; Faulkner, 1988; 1992). Ecological interactions where chemically defended organisms create a refuge from consumers or pathogens are much less common, but have been observed in the marine environment (Hay, 1996; Hay *et al.*, 1998). Symbiotic bacteria have been shown to defend crustacean embryos from a pathogenic fungus (Gil-Turnes *et al.*, 1989); chemically defended algae may provide spatial refuges for herbivores which live on the algae but do not sequester metabolites from their host (Hay *et al.*, 1987; 1990); close associations between chemically defended species and palatable algae have been shown to protect the algae from herbivory (Hay, 1986; Littler *et al.*, 1986; Pfister and Hay, 1988; Kerr and Paul, 1995); an epibiotic sponge has been shown to hinder starfish predation on a spiny oyster (Feifarek, 1987); and a species of Antarctic amphipod has been shown to defend itself from predation by capturing and carrying a chemically defended pteropod (McClintock and Janssen, 1990)

Most of the associational interactions reported thus far are opportunistic rather than coevolved (Hay, 1996). Initially, in the case of *Burnupena papyracea* and *Alcyonidium nodosum*, it could be envisaged that the observed interaction will have also begun as an opportunistic association caused by the unique characteristics of the *B. papyracea* shell making it especially suitable as a substrate for *A. nodosum*. Subsequently, predation by *Jasus* would have given rise to strong selective pressure promoting the symbiotic association between the bryozoan and its host, and it can be considered that the epibiont-whelk associational interaction will have since coevolved, resulting in the intimate relationship observed in the field today.

Burnupena papyracea obviously derives immense benefit from this association. However, whether *Alcyonidium nodosum* gains benefit from the production of the chemical or chemicals involved, and what those benefits may be remains unknown. It is possible that the chemical involved would be produced by *A. nodosum* as a feeding deterrent, although a species of amphipod, *Ochlesis lenticulosus*, has been observed feeding on *Alcyonidium* in the field (Charles Griffiths, pers. com.). It would seem more likely, considering the encrusting nature of this bryozoan, that the active metabolites are produced to inhibit fouling and over-growth of *Alcyonidium*, and this could be tested using field experiments such as those described by Henrikson and Pawlik (1995). The possibility of multiple roles for defensive chemicals in the marine environment has been demonstrated by Becerro *et al.* (1997), so it would not be unreasonable to expect that the chemical or chemicals involved in the *Jasus*-*Burnupena*-*Alcyonidium* interaction may have more than one allelochemic effect. This verisimilitude warrants further detailed study in order to elucidate what benefit is bestowed on the bryozoan by the production of the semiochemical involved in this interaction.

In this investigation, ecologically relevant methods were employed to assess feeding deterrence. Further use of the *Jasus* assay to guide fractionation of the *Alcyonidium* extract in an effort to identify the active components is not, however, feasible. The size and nature of the predator, the duration of the assays, and the large quantities of extract fractions required for testing are simply too prohibitive to facilitate a detailed chemical investigation of the bryozoan extract in the absence of an alternative, reliable bioassay or spectroscopic screening system. The evaluation of other relatively simple and rapid laboratory based bioassays in an attempt to identify a reliable method of directing more extensive chemical investigations of *Alcyonidium nodosum* could be suggested as a worthy area of further research, as this would ultimately allow the deterrent compound or compounds to be characterised and identified. The question here is whether, from an ecological viewpoint, it is actually necessary to identify the chemical responsible for an observed ecological phenomenon: surely establishing and understanding the mechanism of the interaction and identifying the organisms implicated is of more *ecological* importance than knowing the structure of the chemical involved.

Research in marine chemical ecology is currently focused on the use of ecologically relevant bioassays to guide fractionation and isolation of the semiochemicals responsible for various biological interactions in the marine environment (Hay, 1996; Hay *et al.*, 1998). However, I believe that this thesis amply illustrates some of the limitations to this approach by documenting an example where the advantages of using ecologically relevant bioassays must be balanced to some degree against the need for sufficient replication, the variability due to individual differences in behaviour of test organisms, and the statistical methods currently available for analysis of feeding preference experiments.

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Appendix

Bioassay data

Table A.1. Consumption data for *Jasus lalandii* feeding assay 1: encrusted *Burnupena papyracea* versus scraped *B. papyracea* (cells shaded dark grey indicate a rock lobster moult).

Day	Prey	Lobster						Daily	Cumulative
	type	1	2	3	4	5	6	total	total
1	Test	0	1	2	1	0	2	6	6
	Control	1	1	3	0	0	1	6	6
2	Test	0	0	2	0	0	0	2	8
	Control	1	1	1	0	0	1	4	10
3	Test	0	0	2	0	0	0	2	10
	Control	2	1	1	0	0	0	4	14
4	Test	0	0	0	0	1	0	1	11
	Control	0	1	2	1	0	1	5	19
5	Test	0	0	1	0	0	1	2	13
	Control	1	1	2	0	0	0	4	23
6	Test	0	1	2	0	0	0	3	16
	Control	0	1	0	0	0	0	1	24
7	Test	0	0	2	0	0	1	3	19
	Control	0	0	1	0	0	1	2	26
8	Test	0	1	1	0	0	0	2	21
	Control	0	0	2	0	0	0	2	28
9	Test	0	0	0	0	0	0	0	21
	Control	0	1	0	0	0	0	1	29
10	Test	0	0	0	0	0	0	0	21
	Control	0	1	2	0	0	1	4	33
11	Test	0	0	0	0	0	0	0	21
	Control	0	1	2	0	0	0	3	36
12	Test	0	1	0	0	0	0	1	22
	Control	0	1	2	0	0	0	3	39
13	Test	0	1	0	0	0	0	1	23
	Control	0	1	1	0	0	1	3	42
14	Test	0	0	0	0	0	0	0	23
	Control	0	0	2	0	0	0	2	44
Total	Test	0	5	12	1	1	4		
	Control	5	11	21	1	0	6		

Table A.2. Consumption data for *Jasus lalandii* feeding assay 2: pellets containing *Alcyonidium* versus control food pellets (no rock lobsters moulted during this assay).

Day	Prey type	Lobster						Daily total	Cumulative total
		1	2	3	4	5	6		
1	Test	3	0	1	3	0	3	10	10
	Control	3	0	2	3	0	0	8	8
2	Test	2	0	0	0	0	0	2	12
	Control	3	0	0	3	0	0	6	14
3	Test	3	0	0	3	0	0	6	18
	Control	3	0	0	3	0	0	6	20
4	Test	3	0	3	0	0	0	6	24
	Control	3	1	3	0	0	0	7	27
5	Test	0	0	0	2	0	0	2	26
	Control	2	0	0	3	0	0	5	32
6	Test	3	0	0	3	0	0	6	32
	Control	3	0	0	3	0	0	6	38
7	Test	3	0	0	3	0	0	6	38
	Control	3	0	0	3	0	0	6	44
8	Test	3	0	0	3	0	0	6	44
	Control	3	0	0	3	0	0	6	50
9	Test	3	0	0	3	2	2	10	54
	Control	3	0	0	3	2	0	8	58
10	Test	3	0	0	3	0	1	7	61
	Control	3	0	0	3	1	1	8	66
11	Test	3	0	0	3	0	0	6	67
	Control	3	0	0	3	0	0	6	72
12	Test	3	0	0	3	0	0	6	73
	Control	3	0	0	3	0	0	6	78
13	Test	3	1	0	3	2	1	10	83
	Control	3	0	0	3	2	1	9	87
14	Test	3	0	0	3	0	0	6	89
	Control	3	0	0	3	0	0	6	93
Total	Test	38	1	4	35	4	7		
	Control	41	1	5	39	5	2		

Table A.3. Consumption data for *Jasus lalandii* feeding assay 3: encrusted *Burnupena papyracea* versus *B. cincta* (cells shaded dark grey indicate a rock lobster moult).

Day	Prey type	Lobster 1	2	3	4	5	6	Daily total	Cumulative total
1	Test	1	0	1	0	1	0	3	3
	Control	1	0	3	0	3	0	7	7
2	Test	0	0	0	0	0	0	0	3
	Control	1	0	0	0	3	0	4	11
3	Test	0	0	0	0	0	0	0	3
	Control	1	0	0	2	2	0	5	16
4	Test	1	1	0	0	0	0	2	5
	Control	1	0	0	1	3	0	5	21
5	Test	0	0	0	0	0	1	1	6
	Control	1	0	0	1	3	1	6	27
6	Test	1	0	0	0	0	1	2	8
	Control	1	0	0	2	3	0	6	33
7	Test	0	0	0	0	0	0	0	8
	Control	1	0	0	2	3	0	6	39
8	Test	0	0	0	0	0	0	0	8
	Control	3	0	0	3	3	0	9	48
9	Test	0	0	0	0	0	0	0	8
	Control	2	0	1	2	2	0	7	55
10	Test	0	0	0	1	0	0	1	9
	Control	1	0	1	1	1	0	4	59
11	Test	0	0	0	0	0	0	0	9
	Control	3	0	0	3	3	0	9	68
12	Test	1	0	0	0	0	0	1	10
	Control	2	0	0	3	3	0	8	76
13	Test	0	0	0	1	0	0	1	11
	Control	2	0	0	3	2	0	7	83
14	Test	0	0	0	0	0	0	0	11
	Control	1	0	0	0	3	1	5	88
Total	Test	4	1	1	2	1	2		
	Control	21	0	5	22	37	3		

Table A.4. Consumption data for *Jasus lalandii* feeding assay 4: *Burnupena papyracea* encrusted with live *Alcyonidium nodosum* versus *B. papyracea* encrusted with dead *A. nodosum* (cells shaded dark grey indicate a rock lobster moult).

Day	Prey type	Lobster 1	2	3	4	5	6	Total	Cumulative total
1	Test	0	0	0	0	0	0	0	0
	Control	0	0	0	0	0	0	0	0
2	Test	0	0	0	0	0	0	0	0
	Control	0	0	0	0	0	0	0	0
3	Test	2	0	0	0	0	0	2	2
	Control	1	0	2	1	2	1	7	7
4	Test	1	0	0	0	0	0	1	3
	Control	0	0	1	0	2	1	4	11
5	Test	0	0	0	0	0	0	0	3
	Control	0	0	1	0	2	0	3	14
6	Test	0	0	1	1	0	0	2	5
	Control	0	0	0	0	2	0	2	16
7	Test	0	0	1	1	0	0	2	7
	Control	1	0	0	0	1	0	2	18
8	Test	1	0	0	1	0	0	2	9
	Control	1	0	1	1	2	2	7	25
9	Test	0	0	0	0	0	0	0	9
	Control	0	0	0	0	2	1	3	28
10	Test	0	0	0	0	0	1	1	10
	Control	0	0	0	1	1	1	3	31
11	Test	1	0	0	1	0	0	2	12
	Control	0	0	0	0	2	0	2	33
12	Test	1	0	0	1	2	0	4	16
	Control	1	0	0	1	2	0	4	37
13	Test	0	0	0	0	1	0	1	17
	Control	1	0	0	0	3	0	4	41
14	Test	0	0	1	0	0	0	1	18
	Control	1	1	1	0	1	1	5	46
Total	Test	6	0	3	5	3	1		
	Control	6	1	6	4	22	7		

Table A.5. Consumption data for *Jasus lalandii* feeding assay 5: *Burnupena papyracea* encrusted with live *Alcyonidium nodosum* versus *B. papyracea* encrusted with dead *A. nodosum* (cells shaded dark grey indicate a rock lobster moult).

Day	Prey	Lobster							Total	Cumulative
	type	7	8	9	10	11	12	13		total
1	Test	0	0	0	2	0	0	0	2	2
	Control	0	1	2	1	0	0	0	4	4
2	Test	0	0	2	2	1	0	0	5	7
	Control	0	0	0	0	0	0	0	0	4
3	Test	0	0	0	1	0	0	0	1	8
	Control	0	0	0	0	0	0	0	0	4
4	Test	0	0	0	1	0	0	0	1	9
	Control	0	0	0	2	0	0	0	2	6
5	Test	0	1	0	0	0	0	0	1	10
	Control	0	0	0	1	0	0	0	1	7
6	Test	0	0	0	1	0	0	0	1	11
	Control	0	0	0	0	0	0	0	0	7
7	Test	0	0	0	0	0	0	0	0	11
	Control	0	0	0	1	0	0	0	1	8
8	Test	0	0	0	0	0	0	0	0	11
	Control	0	0	0	0	1	0	0	1	9
9	Test	0	0	0	0	0	0	0	0	11
	Control	1	0	0	1	0	0	0	2	11
10	Test	0	0	0	1	0	0	0	1	12
	Control	0	0	0	0	0	0	0	0	11
11	Test	0	0	0	1	0	0	0	1	13
	Control	0	0	0	1	0	0	0	1	12
12	Test	0	0	0	0	0	0	1	1	14
	Control	0	0	0	0	0	0	0	0	12
13	Test	0	0	0	2	0	0	0	2	16
	Control	0	0	0	1	0	0	0	1	13
14	Test	0	0	0	0	0	0	1	1	17
	Control	0	0	1	1	0	0	0	2	15
Total	Test	0	1	2	11	1	0	2		
	Control	1	1	3	9	1	0	0		

Table A.6. Consumption data for *Jasus lalandii* feeding assay 6: encrusted *Burnupena papyracea* versus *B. cincta* (cells shaded dark grey indicate a rock lobster moult).

Day	Prey	Lobster							Total	Cumulative
	type	7	8	9	10	11	12	13		total
1	Test	0	0	0	0	0	0	0	0	0
	Control	0	0	2	1	0	0	2	5	5
2	Test	0	0	0	1	0	0	0	1	1
	Control	0	0	1	0	0	0	1	2	7
3	Test	0	0	0	0	0	0	0	0	1
	Control	0	0	0	0	0	0	0	0	7
4	Test	0	0	0	1	0	0	0	1	2
	Control	0	0	1	0	0	0	0	1	8
5	Test	0	0	0	0	0	0	0	0	2
	Control	0	0	0	0	0	0	0	0	8
6	Test	0	0	0	0	0	0	0	0	2
	Control	0	0	0	0	0	0	1	1	9
7	Test	0	0	0	1	0	0	0	1	3
	Control	0	0	0	0	0	0	0	0	9
8	Test	0	0	0	0	0	0	0	0	3
	Control	0	0	0	0	0	0	1	1	10
9	Test	0	0	0	1	0	0	0	1	4
	Control	0	0	0	0	0	0	0	0	10
10	Test	0	0	0	0	0	0	0	0	4
	Control	0	0	0	0	0	0	0	0	10
11	Test	0	0	0	1	0	0	0	1	5
	Control	0	0	0	0	0	0	0	0	10
12	Test	0	0	0	1	0	0	0	1	6
	Control	0	0	1	0	0	0	0	1	11
13	Test	0	0	0	1	0	0	0	1	7
	Control	0	0	0	0	0	0	0	0	11
14	Test	0	0	0	0	0	0	0	0	7
	Control	0	0	0	0	0	0	0	0	11
Total	Test	0	0	0	7	0	0	0		
	Control	0	0	5	1	0	0	5		

Table A.7. Consumption data for *Jasus lalandii* feeding assay 7: encrusted *Burnupena papyracea* versus scraped *B. papyracea* (no rock lobsters moulted during this assay).

Day	Prey type	Lobster							Total	Cumulative total
		7	8	9	10	11	12	13		
1	Test	0	0	1	1	0	0	0	2	2
	Control	0	0	1	2	0	0	0	3	3
2	Test	0	0	0	0	0	0	0	0	2
	Control	0	0	2	0	0	0	0	2	5
3	Test	0	0	0	0	0	0	0	0	2
	Control	0	0	3	0	0	0	0	3	8
4	Test	0	0	0	0	0	0	0	0	2
	Control	0	0	3	1	1	0	0	5	13
5	Test	0	0	0	0	0	0	0	0	2
	Control	0	0	2	1	1	0	0	4	17
6	Test	0	0	0	0	0	0	0	0	2
	Control	0	0	1	1	1	0	0	3	20
7	Test	0	0	0	1	0	0	0	1	3
	Control	0	0	2	0	1	0	0	3	23
8	Test	0	0	0	1	0	0	0	1	4
	Control	0	0	3	1	0	0	0	4	27
9	Test	0	0	0	1	0	0	0	1	5
	Control	0	0	2	0	0	0	0	2	29
10	Test	0	0	0	1	0	0	0	1	6
	Control	0	0	2	0	0	0	0	2	31
11	Test	0	0	0	1	0	0	0	1	7
	Control	0	0	1	1	0	0	0	2	33
12	Test	0	0	0	0	0	0	0	0	7
	Control	0	0	1	1	0	0	0	2	35
13	Test	0	0	0	1	0	0	0	1	8
	Control	0	0	1	1	2	0	0	4	39
14	Test	0	0	0	0	0	0	0	0	8
	Control	0	0	0	1	3	0	0	4	43
Total	Test	0	0	1	7	0	0	0		
	Control	0	0	24	10	9	0	0		

Table A.8. Consumption data for *Jasus lalandii* feeding assay 8: pellets containing *Alcyonidium* versus control food pellets (no rock lobsters moulted during this assay).

Day	Prey type	Lobster							Total	Cumulative total
		7	8	9	10	11	12	13		
1	Test	3	3	3	3	3	0	3	18	18
	Control	3	3	3	3	3	0	3	18	18
2	Test	0	2	2	2	3	0	3	12	30
	Control	3	3	2	2	3	3	3	19	37
3	Test	1	1	0	0	1	0	2	5	35
	Control	3	3	1	2	3	3	3	18	55
4	Test	0	0	0	0	3	0	2	5	40
	Control	3	0	0	0	3	3	3	12	67
5	Test	0	0	0	0	3	1	1	5	45
	Control	3	1	2	1	3	2	3	15	82
6	Test	0	1	1	0	3	0	1	6	51
	Control	2	1	2	1	3	2	2	13	95
7	Test	0	0	2	0	3	2	0	7	58
	Control	3	0	2	0	3	3	3	14	109
8	Test	0	0	2	0	3	2	0	7	65
	Control	2	1	1	2	3	2	2	13	122
9	Test	0	0	2	0	3	3	1	9	74
	Control	3	0	2	1	3	3	3	15	137
10	Test	0	0	0	1	1	0	0	2	76
	Control	2	1	1	1	1	0	1	7	144
11	Test	0	0	1	0	3	0	0	4	80
	Control	3	1	2	1	3	3	3	16	160
12	Test	3	0	0	0	0	3	0	6	86
	Control	3	0	0	1	3	3	3	13	173
13	Test	3	0	0	0	1	1	0	5	91
	Control	3	0	0	0	2	3	0	8	181
14	Test	3	1	0	0	1	3	0	8	99
	Control	3	1	1	0	3	3	3	14	195
Total	Test	13	8	13	6	31	15	13		
	Control	39	15	19	15	39	33	35		

Table A.9. Consumption data for *Jasus lalandii* feeding assay 9: pellets containing *Alcyonidium* versus control food pellets (no rock lobsters moulted during this assay).

Day	Prey type	Lobster												Total	Cumulative total
		14	15	16	17	18	19	20	21	22	23	24	25		
1	Test	0	3	1	3	0	3	2	3	0	0	2	0	17	17
	Control	1	3	0	3	0	3	3	3	0	0	3	0	19	19
2	Test	0	0	0	2	0	1	1	3	1	0	3	0	11	28
	Control	1	3	0	3	1	3	3	3	1	1	3	2	24	43
3	Test	0	1	0	1	0	1	1	3	1	0	2	0	10	38
	Control	0	3	1	2	1	1	3	3	0	0	3	1	18	61
4	Test	0	0	1	1	0	1	1	0	1	0	0	0	5	43
	Control	1	2	1	3	2	2	3	1	2	1	2	1	21	82
5	Test	0	0	0	1	0	3	1	3	0	0	3	3	14	57
	Control	0	0	2	3	0	3	3	3	0	0	3	3	20	102
6	Test	0	0	1	1	0	0	0	3	0	1	2	3	11	68
	Control	0	0	3	3	1	3	3	3	0	3	3	3	25	127
7	Test	0	0	0	2	0	1	1	3	0	3	3	3	16	84
	Control	0	0	2	3	0	3	3	3	1	3	3	3	24	151
8	Test	1	0	0	1	0	0	1	2	0	1	2	1	9	93
	Control	2	1	1	3	0	2	2	3	0	2	2	2	20	171
9	Test	0	0	0	0	0	0	1	1	0	0	1	0	3	96
	Control	1	0	2	0	1	2	1	2	1	2	3	1	16	187
10	Test	0	0	0	1	0	0	0	3	0	3	0	0	7	103
	Control	0	0	0	3	0	3	3	3	1	3	3	3	22	209
11	Test	0	0	0	0	0	0	0	3	0	3	0	3	9	112
	Control	0	0	3	3	0	3	3	3	0	3	3	3	24	233
12	Test	0	0	0	0	0	0	0	0	0	3	0	3	6	118
	Control	2	1	3	3	0	3	3	3	0	3	3	3	27	260
13	Test	0	0	0	1	0	0	0	0	0	3	0	3	7	125
	Control	0	0	3	3	3	3	3	3	0	3	3	3	27	287
14	Test	0	0	0	1	0	0	0	0	0	3	0	3	7	132
	Control	0	0	3	3	2	3	3	3	0	3	3	3	26	313
Total	Test	1	4	3	15	0	10	9	27	3	20	18	22		
	Control	8	13	24	38	11	37	39	39	6	27	40	31		

Table A.10. Consumption data for *Jasus lalandii* feeding assay 10: pellets containing *Alcyonidium* extract versus control food pellets (no rock lobsters moulted during this assay).

Day	Prey	Lobster												Total	Cumulative
	type	14	15	16	17	18	19	20	21	22	23	24	25		total
1	Test	1	1	0	2	0	3	3	3	3	1	0	0	17	17
	Control	1	3	0	3	0	3	3	3	3	3	0	0	22	22
2	Test	0	0	0	3	0	1	3	3	2	0	0	0	12	29
	Control	2	2	1	3	0	3	3	3	3	3	1	1	25	47
3	Test	0	1	0	3	0	2	3	0	1	0	0	0	10	39
	Control	1	3	0	3	0	3	3	3	2	2	1	0	21	68
4	Test	0	0	0	2	0	2	3	0	1	0	0	2	10	49
	Control	0	1	0	3	1	2	3	2	2	3	1	1	19	87
5	Test	1	2	1	1	0	1	3	1	3	1	0	0	14	63
	Control	2	3	0	3	0	3	3	3	2	2	1	0	22	109
6	Test	0	2	0	2	0	3	3	1	0	0	0	0	11	74
	Control	3	3	0	3	0	3	3	3	3	2	1	2	26	135
7	Test	0	0	0	3	0	1	3	1	0	0	0	0	8	82
	Control	2	3	1	3	0	3	3	0	1	1	1	1	19	154
8	Test	1	0	0	2	0	0	3	0	0	0	0	0	6	88
	Control	3	2	0	3	1	3	3	1	2	0	1	0	19	173
9	Test	1	2	0	0	0	1	1	0	0	0	0	0	5	93
	Control	1	3	0	3	0	2	3	0	1	0	3	1	17	190
10	Test	0	0	0	2	0	1	3	1	0	1	0	0	8	101
	Control	3	1	0	3	1	2	3	2	3	0	1	0	19	209
11	Test	1	2	0	3	0	3	1	0	1	0	0	0	11	112
	Control	3	3	1	3	0	3	3	2	2	0	0	0	20	229
12	Test	0	3	0	3	0	2	0	0	0	0	0	0	8	120
	Control	3	3	1	3	0	3	3	2	2	0	2	0	22	251
13	Test	1	0	0	2	0	1	0	0	1	1	1	0	7	127
	Control	3	3	1	3	1	3	3	2	1	1	3	0	24	275
14	Test	0	0	0	0	0	0	1	0	0	0	0	0	1	128
	Control	3	3	1	2	2	1	2	2	1	1	2	2	22	297
Total	Test	6	13	1	28	0	21	30	10	12	4	1	2		
	Control	30	36	6	41	6	37	41	28	28	18	18	8		

Table A.11. *Artemia salina* larvicidal bioassay data: mortality data for *Alcyonidium nodosum* extracts and solvent controls (OCE = organic crude extract, HSE = hexane soluble extract, MSE = methanol soluble extract).

Concentration (µg/ml)	Replicate	OCE			HSE			MSE			Control		
		Number responding	Total number exposed	Percentage mortality	Number responding	Total number exposed	Percentage mortality	Number responding	Total number exposed	Percentage mortality	Number responding	Total number exposed	Percentage mortality
400	1	0	16	0	0	16	0	1	15	7	2	20	10
	2	1	21	5	0	16	0	2	23	9	0	16	0
	3	0	14	0	0	18	0	2	24	8	3	31	10
	4	0	24	0	0	15	0	0	16	0	1	15	7
	5	0	15	0	1	22	5	0	28	0	0	18	0
	6	1	32	3	0	21	0	3	29	10	0	14	0
200	1	0	17	0	0	19	0	1	18	6	1	25	4
	2	1	18	6	0	18	0	0	17	0	1	38	3
	3	2	15	13	0	20	0	1	19	5	1	14	7
	4	0	18	0	0	16	0	0	17	0	0	27	0
	5	0	16	0	1	24	4	0	18	0	0	18	0
	6	0	18	0	0	18	0	1	20	5	0	14	0
100	1	0	15	0	1	18	6	1	16	6	0	20	0
	2	1	16	6	0	18	0	1	14	7	0	19	0
	3	3	20	15	0	20	0	0	24	0	0	24	0
	4	0	18	0	0	21	0	0	21	0	0	21	0
	5	0	14	0	0	27	0	1	15	7	0	22	0
	6	0	27	0	0	26	0	0	30	0	0	17	0
50	1	0	17	0	0	23	0	0	20	0	0	23	0
	2	0	21	0	0	13	0	1	24	4	0	16	0
	3	1	21	5	0	16	0	0	18	0	0	13	0
	4	0	19	0	0	15	0	0	17	0	0	13	0
	5	0	18	0	0	21	0	0	15	0	1	16	6
	6	0	18	0	0	11	0	0	19	0	0	23	0