Chemical ecology and palatability of marine invertebrates in the sub-Arctic Kongsfjord (Spitsbergen)

Chemische Ökologie und Nahrungsattraktivität mariner Evertebraten im Sub-Arktischen Kongsfjord (Spitzbergen)

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Die vorliegende Arbeit ist die inhaltlich leicht veränderte Fassung einer kumulativen Dissertation, die in der Sektion „Vergleichende Ökosystemforschung“ bei Prof. Dr. Wolf E. Arntz angefertigt und 2003 dem Fachbereich 2 (Biologie/Chemie) der Universität Bremen vorgelegt wurde.

CONTENTS

SUMMARY

While the development of chemical defenses in marine organisms of tropical and Antarctic regions is supposed to be driven by intense pressure of predation and competition, the selective pressure for chemical defenses has been generally proposed to be less important in northern high latitudes. However, predator-prey relationships are still little understood for Arctic and sub-Arctic hard substrate communities. Additionally, many invertebrate species in temperate, tropical and Antarctic regions have chemical defenses against the formation of microbial, and possibly deleterious, surface films. There is no reason why the selective pressure exerted by microorganisms should be lower on Arctic invertebrates. Therefore, the overall aim of this study was to assess for the first time the incidence of chemical defenses against predation and microbial colonization in 18 abundant sessile or slow moving invertebrates from the sub-Arctic Kongsfjord (Spitsbergen).

To investigate the palatability of body tissue of invertebrate species and their value as a food source, *in situ* experiments were performed with natural consumer assemblages in the Kongsfjord. These experiments were complemented with quantitative laboratory assays, using the generalist predatory starfish *Asterias rubens* from the North Sea. Feeding preference and avoidance reactions were similar in both assays. Natural assemblages of predators *in situ* rejected nine out of ten species tested, and twelve out of sixteen species were unpalatable in laboratory assays. Results of both assays were compared to the biochemical composition of the investigated species to see whether palatability and feeding preferences coincide with nutritional quality of the prey. Nutritional quality, expressed as protein, lipid, nitrogen, carbon and water content, may account for some of the feeding preferences found, but no overall relationship between nutritional value and palatability or feeding preferences was detected.

The feeding deterrent activity of crude extracts of the investigated invertebrates was tested to assess the incidence of chemical defense against

predation. Laboratory assays were performed by offering artificial food with and without extracts to two different consumers, the amphipod *Anonyx nugax* which is a common species in Kongsfjord, and the starfish *Asterias rubens* from the North Sea. Two of the eighteen extracts tested (sponge *Haliclona viscosa*, actinia *Hormathia nodosa*), exhibited significant feeding deterrent effects in the amphipod assay. Furthermore, six extracts had a significantly stimulating effect on the amphipod feeding, and ten extracts did not affect consumption by the amphipods compared to control food. In the starfish assay only the crude extract of *Haliclona viscosa* was significantly rejected. For *Haliclona viscosa*, feeding deterrence could be traced back to two different compounds, and for *Hormathia nodosa* to one fraction.

Invertebrate crude extracts were also tested for their inhibitory effects on the growth of five co-occurring phylogenetically diverse bacterial strains. Six out of eighteen (33%) crude extracts inhibited bacterial growth at natural extract concentrations. The crude extract of the sponge *Haliclona viscosa* inhibited growth of all five bacterial strains, suggesting the presence of metabolites with broad-spectrum activity. Three active compounds were isolated from *H. viscosa* but no synergistic effects were detected among these three compounds.

The sponge *Haliclona viscosa* is a particularly chemically rich species, since two different feeding deterrent compounds and two additional metabolites with antimicrobial activity are present. The chemical structures of three active compounds have been identified to be closely related to known alkaloids from other sponges.

The present data indicate that feeding deterrent and antibacterial secondary metabolites are present in sub-Arctic marine invertebrates but are less abundant than in temperate, tropical, or Antarctic species. At this point it seems that predation is not strong enough selective pressure to drive the development of chemical defenses in a large variety of invertebrates. This would coincide with the latitudinal hypothesis, which predicts an inverse relationship between chemical defense and latitude with a lower incidence of chemical defense in higher latitudes. However, more chemical ecology studies from other, particularly high Arctic locations, are needed to draw conclusions about selective adaptations in Arctic benthic communities.

ZUSAMMENFASSUNG

Die evolutive Entwicklung von chemischen Abwehrsubstanzen in marinen Organismen der Tropen und der Antarktis wird überwiegend auf einen intensiven Selektionsdruck durch Prädation und Konkurrenz zurückgeführt. Im Gegensatz dazu wird diesen Faktoren in hohen nördlichen Breiten generell eine geringe Bedeutung zugeschrieben. Räuber-Beute-Beziehungen in arktischen und sub-arktischen Hartsubstrat-Gemeinschaften sind insgesamt gesehen jedoch noch relativ wenig untersucht. Zudem zeigen zahlreiche Evertebraten in gemäßigten, tropischen und antarktischen Meeresregionen chemische Schutzstrategien gegen mikrobielle Oberflächenfilme, die sich potentiell schädlich auf die Tiere auswirken können. Auch wenn entsprechende Studien fehlen, kann davon ausgegangen werden, daß der durch Mikroorganismen ausgeübte Selektionsdruck in der Arktis ähnlich sein sollte. Das Hauptziel dieser Arbeit war daher die erstmalige Untersuchung von chemischen Abwehrsubstanzen gegen Räuber und Bakterien in 18 abundanten, sessilen oder sich langsam fortbewegenden Evertebraten aus dem sub-arktischen Kongsfjord (Spitzbergen).

Zur Untersuchung der Attraktivität der Evertebraten als Nahrungsquelle wurden in Freilandversuchen natürlichen Konsumentenpopulationen Gewebestücke der Tiere angeboten. Diese Experimente wurden durch quantitative Laborversuche mit dem Seestern *Asterias rubens* aus der Nordsee ergänzt. Die Fraß- und Ablehnungreaktionen waren in beiden Experimenten ähnlich. In den Freilandversuchen wurden von zehn angebotenen Arten neun von den Prädatoren abgelehnt, in den Laborexperimenten wurden zwölf von sechzehn Arten nicht gefressen. Die Ergebnisse beider Versuche wurden mit dem Nährwert der untersuchten Evertebraten verglichen, um Korrelationen zwischen Beuteattraktivität und Nahrungsqualität festzustellen. Die Nahrungsqualität wurde als Protein-, Lipid-, Stickstoff-, Kohlenstoff- und Wassergehalt bestimmt, und kann einige der beobachteten Nahrungspräferenzen erklären. Ein allgemeiner Zusammenhang zwischen dem Nährwert und der Attraktivität der Evertebraten als Futter konnte jedoch nicht nachgewiesen werden.

Die fraßhemmende Wirkung von Rohextrakten der untersuchten Tiere wurde getestet um die Häufigkeit des Auftretens von chemischen Fraßschutzsubstanzen festzustellen. Zwei verschiedenen Konsumenten, dem im Kongsfjord häufigen

Amphipoden *Anonyx nugax* und dem Seestern *Asterias rubens* aus der Nordsee wurde in Laborversuchen künstliches Futter mit und ohne Extrakt angeboten. Von den 18 getesteten Extrakten erwiesen sich in den Versuchen mit *A. nugax* zwei Tiere, der Schwamm *Haliclona viscosa* und die Anthozoe *Hormathia nodosa*, im Vergleich zum Kontrollfutter als signifikant fraßhemmend. Zudem hatten sechs Extrakte anderer Evertebraten eine fraßstimulierende Wirkung, während zehn Extrakte keinen Einfluß auf den Amphipodenfraß zeigten. Die fraßhemmende Wirkung des *H. viscosa* Rohextraktes konnte auf zwei verschiedene Substanzen zurück geführt werden, die von *H. nodosa* auf eine Fraktion.

Zudem wurde die Wirkung der Evertebraten-Rohextrakte auf das Wachstum von fünf sympatrischen, phylogenetisch unterschiedlichen Bakterienstämmen untersucht. Sechs von achtzehn Rohextrakten zeigten eine hemmende Wirkung auf das Bakterienwachstum. Darunter beeinträchtigte als einziger der Rohextrakt aus *H. viscosa* das Wachstum aller fünf Bakterienstämme, was auf das Vorhandensein von Sekundärmetaboliten mit breitem Wirkungsspektrum deutet. Insgesamt wurden drei antibakteriell aktive Substanzen aus *H. viscosa* isoliert. Synergistische Effekte zwischen den Substanzen wurden jedoch nicht festgestellt.

Haliclona viscosa hat sich als eine chemisch besonders reiche Art herausgestellt, da zwei fraßhemmende Substanzen und zusätzlich zwei Komponenten mit antibakterieller Wirkung nachgewiesen wurde. Die chemische Struktur von drei dieser Metabolite wurde inzwischen aufgeklärt. Sie sind nahe verwandt mit bereits bekannten Alkaloiden aus anderen Schwämmen.

Die vorliegenden Daten zeigen, daß fraßhemmende und antibakterielle Sekundärmetabolite in sub-arktischen marinen Evertebraten vorkommen, jedoch in geringerem Maße als in Arten aus gemäßigten, tropischen oder antarktischen Gebieten. Demnach scheint Prädation keinen ausreichend starken Selektionsdruck auf die Entwicklung einer chemischen Verteidigung in einer großen Anzahl von Evertebraten des Kongsfjordes auszuüben. Dies würde mit der "Latitudinal Hypothesis" übereinstimmen, die eine inverse Beziehung zwischen chemischer Verteidigung und dem Breitengrad postuliert, mit einer geringeren Häufigkeit chemischer Schutzmechanismen in höheren Breiten. Es sind jedoch weitere Untersuchungen aus anderen, besonders hocharktischen Gebieten zur chemischen Ökologie notwendig, bevor Schlußfolgerungen über selektive Anpassungen in arktischen Benthos-Gemeinschaften gezogen werden können.

1 INTRODUCTION

1.1 MARINE CHEMICAL ECOLOGY

Interactions between organisms can significantly structure species composition and density in a marine ecosystem. These interactions may occur between different species or between individuals of the same species, including competition for space, light, nutrients or food, parasitism, symbiosis and reproduction. During the last decades it has been shown that interactions are often chemically mediated by so-called secondary metabolites or natural products (Bakus et al. 1986; Hay 1996; McClintock & Baker 1997; Amsler et al. 2001; Paul et al. 2001; Steinberg & de Nys 2002). For a long time these secondary metabolites have been regarded as metabolic waste products or compounds of primary metabolic overflow without any obvious biological function. Today there is increasing evidence that these substances play fundamental roles in ecology, among others as pheromones, feeding deterrents, mediators of spatial competition, site recognition or homing cues, inhibitors of fouling and infection or UV-sunscreens, and in the facilitation of reproduction (Harper et al. 2001). Consequently, secondary metabolites may contribute as much as primary metabolites to the survival of the producing organism. While primary metabolites such as amino acids, carbohydrates and fatty acids are chemically almost identical in most organisms, one characteristic of secondary metabolites is their high chemical diversity and their limited phylogenetic distribution. Major structural classes of natural products include terpenes, alkaloids, polyketides, peptides, and shikimate-derived metabolites, as well as compounds of mixed biogenesis (Dietzman 1997; Faulkner 2000).

Marine chemical ecology is a relatively young field. While natural products from terrestrial plants and microorganisms have already been isolated since the 1930s, natural compounds mediating interactions between marine invertebrates have only recently been investigated during the past 25 years, partly due to the development of underwater technology and the establishment of ecologically

relevant bioassays (McClintock & Baker 2001). To date most commonly investigated in the marine environment are predator-prey relationships and the feeding deterrent effects of secondary metabolites (Harper et al. 2001). Particularly soft bodied, sessile or sluggish organisms such as sponges, ascidians, actinians, soft corals, and nudibranchs appear to be physically vulnerable to predation and probably have little ability to actively avoid predation (Chanas & Pawlik 1996). Many of these invertebrates possess chemical defenses against potential predators (Faulkner & Ghiselin 1983; Sammarco & Coll 1992; Van Alstyne & Paul 1992; Schupp et al. 1999; Assmann et al. 2000; Puglisi et al. 2000; Amsler et al. 2001). Besides these deterrent effects against predators, chemical defenses against colonization by microorganisms, algae and invertebrates represent an intensely studied field (Bergquist & Bedford 1978; Davis et al. 1989; Wahl 1989; Sammarco & Coll 1992; Henrikson & Pawlik 1995; Newbold et al. 1999; Steinberg et al. 2001).

Quantitative variations in secondary metabolites involved in chemical defenses have been found between different tissues of an individual (Schupp et al. 1999), between different populations (Swearingen & Pawlik 1998), and on large geographical scales (Harvell et al. 1993; Bolser & Hay 1996; Puglisi et al. 2000). Chemical defense is generally supposed to be costly at the expense of other functions (e.g. growth, reproduction) and to be developed upon selective pressure (Barnes & Hughes 1988; Herms & Matteson 1992; Berenbaum 1995). Thus, it can be assumed that chemical defenses should only be produced when the organism gains benefit. The distribution of secondary metabolites among species and regions therefore provides insight into factors driving ecological specialisation and is thereby fundamental to a wide range of ecological and evolutionary topics (Hay 1992). To date, extensive data on marine chemical ecology in tropical regions and several recent studies from Antarctica exist but still little is known from temperate regions and virtually nothing from Arctic waters.

- Chemical ecology aims to explain interactions between organisms on a chemical basis.
- Secondary metabolites are involved in mediating a diverse array of inter- and intraspecific interactions including predation, competition, symbiosis and reproductive processes.

- Secondary metabolites are supposed to have developed under evolutionary selective pressure.
- Nothing is known so far on the chemical ecology from Arctic and sub-Arctic organisms.

1.2 APPLIED ASPECTS

Beside the ecological aspects of natural products mentioned above, chemical ecology represents a particular interesting field for applied research, mainly for the search and discovery of new pharmaceutical drugs. Today 57% of the 150 most prescribed drugs have their origin in natural products (Wallace 1997). Marine metabolites have been proved to function, among others, as anitbiotics, pain suppressers, anti-inflammatory agents, skin care products, sun screens, and anticancer agents (Munro et al. 1999).

Additionally to the pharmaceutical applications of marine natural products the search for new nontoxic marine antifouling coatings has gained increasing interest during the last years. Settlement of marine invertebrates or macroalgae to the hulls of ships is one of the most important problems facing marine technology (Hattori & Shizuri 1996). Fouling causes an increase of drag, which leads to a decrease in speed, maneuverability and fuel efficiency and thereby to an increase of costs. Antifouling coatings are mainly based on broad-spectrum biocides, usually toxic metal ions such as tributyltin (TBT), and have deleterious environmental impacts (Rittschof 2001). Nontoxic marine natural products without associated environmental problems may potentially provide an alternative to heavy metal-based paints (Kjelleberg & Steinberg 1994).

- Chemical ecology has ecological as well as applied aspects.
- Marine natural products provide a potential source of new structures for pharmaceutical drugs and nontoxic antifouling coatings.

1.3 THE LATITUDINAL HYPOTHESIS

In 1974 Bakus & Green (Bakus 1974; Bakus & Green 1974; Green 1977) suggested an inverse relationship between latitude and the incidence of chemical defense, with the highest incidence of chemical defenses at low latitudes, particularly in the tropics. At lower latitudes, high biodiversity and the resulting influence of biological interactions such as predation and competition in benthic communities have been suggested to drive the development of chemical defenses in sessile and slow moving invertebrates. Mainly the intense predation by fishes in tropical regions is thought to have resulted in increased selection for noxious and toxic chemical compounds in tropical marine invertebrates (Bakus 1974; Bakus & Green 1974; Green 1977). More recently, this latitudinal hypothesis has come into question, and several authors showed that at least for the southern hemisphere this relationship is invalid (McClintock & Baker 1997; Avila et al. 2000; Amsler et al. 2001). The incidence of toxicity in Antarctic sponges, for example, is similar to that in tropical and much higher than in temperate species (McClintock 1987). Already Dayton et al. (1974) suggested that Antarctic benthic communities are "biologically accommodated" and are structured mainly by biological factors such as predation and competition, favored by the stable physical conditions below the zone of ice scour.

Benthic communities in northern high latitudes are commonly considered to be less diverse than those from lower latitudes, especially from tropical regions, but also from Antarctica (Thorson 1957; Kendall 1996; Arntz et al. 1997; Starmans et al. 1999; Gray 1997, 2001). Not all investigations, however, confirm this pattern and, locally, Arctic communities can be rich in biomass and diversity (Kendall & Aschan 1993; Kendall 1996; Mayer & Piepenburg 1996; Brandt 1997). In contrast to the Antarctic, Arctic and sub-Arctic benthic communities are supposed to be mainly structured by physical factors, such as wave action, ice gouging (Barnes et al. 1984; Woodworth-Lynas et al. 1991; Gutt et al. 1996), activity of bottom feeding fish and mammals (Nerini & Oliver 1983; Johnson & Nelson 1984; Oliver et al. 1985; Dayton et al. 1994), and the influence of glaciers and river runoffs, adding high loads of inorganic material and freshwater mainly to inner-fjord locations (Gulliksen et al. 1985; Wlodarska et al. 1996; Wlodarska-Kowalczuk et al. 1998). Additionally, it is suggested that the relatively young evolutionary history of the

Arctic Ocean (Dayton et al. 1994) contributes to a low biodiversity due to a relatively short period for adaptation and speciation (Gray 2001).

Although Thorson (1957) and Gulliksen (1979) found predation on benthic communities to be sparse in Arctic regions, locally, predation by walruses (Oliver et al. 1985) or gray whales (Nerini & Oliver 1983; Highsmith & Coyle 1992) can have a significant impact. Among lower trophic levels predator-prey relationships are relatively unstudied in Arctic waters, especially within the less abundant hard bottom communities. In the Kongsfjord on Spitsbergen larger areas of colonized hard substrate are restricted to few sites, mainly Hansneset in the middle and Kongsfjordneset in the outer part of the fjord (Fig. 1). These locations are exposed to wave action and currents, and can be densely populated by sessile filter feeders which may represent up to 90% of the standing biomass at depth below 15-20 m (Hop et al. 2002). Beside these restricted locations, also ice rafted drop stones, which are common throughout the fjord (Whittington et al. 1997), provide a substratum for the development of sessile hard bottom communities. Although such hard bottom communities are patchily distributed throughout the Kongsfjord, their high accumulations of invertebrates may provide an important feeding ground for epibenthic predators in an otherwise soft bottom dominated habitat. If food sources are highly limited then even a few abundant predators may still create strong feeding pressure. Consequently, the hard bottom community patches of high biomass and diversity could also be regarded as areas of high biological activity, and biological interactions such as predation may play an important role in structuring these communities. Sub-Arctic invertebrates may therefore well have been under selective pressure for the development of chemical defenses against predation.

Predation is just one factor exerting selective pressure on marine invertebrates. Another one would be the fouling of epibiontic organisms. All multicellular organisms in the marine environment are virtually bathed in a ‚microbial soup' (Jenkins et al. 1998), and any solid living or non-living surface is exposed to colonization by bacteria. Bacterial surface conditioning and colonization have been considered to be the first stages of surface fouling, promoting subsequent settlement of unicellular and multicellular eukaryotic organisms (Wahl 1989). Filter-feeding animals, such as sponges, that feed on bacteria (Berquist 1978) concentrate microorganisms from the water column

during the feeding process and are thus additionally exposed to high quantities of microbes including potential pathogens. Total bacterial numbers in cold waters are proposed to be generally decreased compared to lower latitudes, and it could be hypothesized that organisms would invest less resources into antimicrobial defense when the threat of bacterial colonization is lower. However, water column bacterial numbers alone do not necessarily reflect the selective pressure exerted on marine invertebrates, and locally microbial cell density can be increased, for example, at Arctic inner fjord locations due to a high amount of particulate organic carbon at glacial meltwater outflows (Jankowska & Wlodarska-Kowalczuk, in prep.).

Consequently, from the prevailing environmental conditions it cannot be excluded that Arctic and sub-Arctic marine invertebrates have been under selective pressure for the development of chemical defenses against predation and detrimental surface colonizing or pathogenic micoorganisms. So far the latitudinal hypothesis has not been tested in northern high latitudes. In the present study chemically mediated interactions between marine invertebrates in a sub-Arctic region are investigated for the first time.

- the latitudinal hypothesis predicts an inverse relationship between latitude and the incidence of chemical defense
- organisms in lower latitudes (tropics) are supposed to be more chemically defended that organisms in higher latitudes
- the latitudinal hypothesis has proven invalid for the Antarctic, nothing is known about high northern latitudes
- Arctic benthic communities are supposed to be structured by physical rather than biological processes

⇒ **Hypothesis 1**

- Patchily distributed benthic hard bottom communities of high bioactivity in the sub-Arctic Kongsfjord can be under high selective pressure by even few abundant predators and by microorganisms.
- Northern high latitude invertebrates are chemically defended
- The latitudinal hypothesis is invalid for Arctic waters

1.4 DEFENSES OTHER THAN SECONDARY METABOLITES

Defenses against predators as well as against detrimental surface colonizing or pathogenic microorganisms do not have to be chemical. Marine organisms have developed a range of defensive mechanisms including behavioural and physical strategies. Surface fouling or microbial colonization can be reduced by mechanical processes like tissue or mucus sloughing (Krupp 1985; Barthel & Wolfrath 1989), by physical properties like surface tension (Becker & Wahl 1991) or by surface acidity (Hirose et al. 2001). Also against predation the defensive options available to marine organisms are diverse, including low nutritional quality (McClintock 1987; Duffy & Paul 1992; Chanas & Pawlik 1995; Granado & Caballero 2001), structural defenses (Steneck & Watling 1982; Littler et al. 1983; Harvell 1984; Van Alstyne & Paul 1992; Chanas & Pawlik 1995; Koh et al. 2000; Puglisi et al. 2000), tissue toughness (Chanas & Pawlik 1995), temporal or spatial avoidance of predators (Rogers et al. 1995). Many organisms use two or several of these strategies in combination, but investigations of synergistic effects between different defense mechanisms are rare (Hay et al. 1994; Pennings 1996). Duffy & Paul (1992) showed that, for example, the nutritional quality and the chemical defense of a prey in concert determine its susceptibility to a consumer. In their study these authors offered food differing in concentration of proteins and carbohydrates to predatory reef fish and found that some compounds were effective at defending low protein foods but ineffective at defending higher protein foods. Consequently, for the understanding of the outcome of predator prey interactions it is essential to regard also other defensive mechanisms than just secondary metabolites. A second objective of the present study, therefore, was to examine the nutritional quality of benthic invertebrates, measured as protein, lipid, nitrogen and water content, to estimate their potential as a prey.

- The defensive options available to marine organisms are diverse including behavioural and physical strategies as well as being of low nutritional quality.
- The different defenses may act synergistically.

⇒ **Hypothesis 2**

Low nutritional quality in sub-Arctic benthic invertebrates serves as defense against predators

1.4 AIMES OF THE PRESENT STUDY

To verify the hypothesis that invertebrates from northern high latitudes are defended against predators and detrimental microorganisms the aims of the present study were

(a) to assess the palatability of conspicuous sessile or sluggish members of shallow subtidal (≤ 30 m) hard bottom communities in a sub-Arctic ecosystem

(b) to examine the nutritional quality of the same benthic invertebrates, measured as protein, lipid, nitrogen and water content, to estimate their potential as a prey

(c) to investigate potential chemical defenses of these invertebrates against predators

(d) to assess the chemical antimicrobial properties of the invertebrates on co-occurring microorganisms

2 STUDY SITE

2.1 KONGSFJORD

Kongsfjord, where the present study was carried out, is situated at the northwestern coast of Spitsbergen at 79° N and 12° E (Fig. 1). The fjord is 26 km long, has a width between 3 and 8 km, and a maximum depth of 400 m. The tidal range is about 2 m with weak currents (Ito & Kudoh 1997). During the summer season four glaciers and a number of glacier run-offs add terrestrial sediments (Elverhøi et al. 1983) and freshwater to the fjord, which may reduce salinity locally from an average of 34 down to 20 PSU. The annual mean water temperature is slightly above 0°C (Ito & Kudoh 1997), however, in summer maximum temperatures of about 6°C at the surface and of about 4°C at 20 m depth are measured (Hanelt et al. 2001).

The west coast of Spitsbergen is influenced by the West Spitsbergen Current, which is composed of the northern-most extension of the North Atlantic Current, carrying relatively warm and salty water, and of the cooler and less salty water masses from the Jan Mayen and East Spitsbergen currents. Since Kongsfjord is an open fjord without a sill at the entrance it is influenced by glacial input as well as by oceanic water (Svendsen et al. 2002). Because of this Atlantic influence the fjord is to be regarded as sub-Arctic rather than Arctic, despite its location at high latitude (Hop et al. 2002).

Based on zoogeographical composition western Spitsbergen is classified as a transition zone between Arctic and boreal regions (Wlodarska-Kowalczuk et al. 1998). Large areas of Kongsfjord (below 5-10 m depth), particularly in its inner basin, are composed of poorly consolidated soft mud deposits from the outflow of adjacent glaciers (Hop et al. 2002). High sedimentation rates, ice scouring and freshwater influence lead to a depauperate epibenthic fauna in these soft sediment areas (Curtis 1975; Elverhøi et al. 1983; Piepenburg et al. 1996; Wlodarska-Kowalczuk et al. 1998). Hard substrate is found in many intertidal areas down to around 4 m depth but is mostly uncolonized by invertebrates due to ice abrasion. Larger areas of densely populated hard substrate are restricted to few sites, mainly Hansneset in the middle and Kongsfjordneset in the outer part of the fjord (Fig. 1). Beside these restricted locations, also ice rafted drop stones, which are

common throughout the fjord (Whittington et al. 1997), provide a substratum for the development of sessile bottom communities.

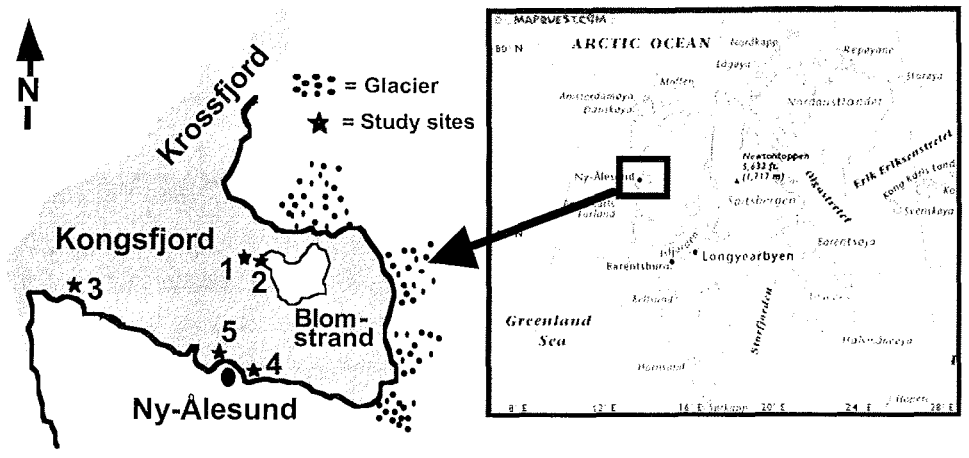
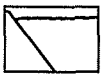
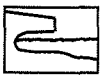
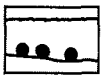


Figure 1: Spitsbergen, Kongsfjord; for sites 1-4 see Table 1, site 5 is location of field experiments (see section 3.4.1).

2.2 SAMPLING SITES IN THE KONGSFJORD

Invertebrates were collected at four different locations in Kongsfjord. A detailed description of the different sites can be found in publication I. Table 1 gives an overview on topography, substrate and abundant fauna at the different study sites.

Table 1: Description of the different sampling sites (locations see Fig. 1)

Site	Topography	Substrate	Fauna
1: Hansneset		rocky	actinians, ascidians, bryozoans, sponges, bivalves, barnacles
2: Kongsfjordneset		rocky	actinians, ascidians, bryozoans, sponges, bivalves, barnacles
3: Cave		rocky	octocoralls, sponges, bryozoans, actinians, ascidians
4: Inner fjord		soft sediment with single bolders	ascidians, bryozoans, barnacles

3 MATERIAL AND METHODS

An overall view on the methods employed in the present study is given in Figure 2. In the following, I will briefly summarize the methodological principles while a more detailed description can be found in publications I, II and III, chapter 6.

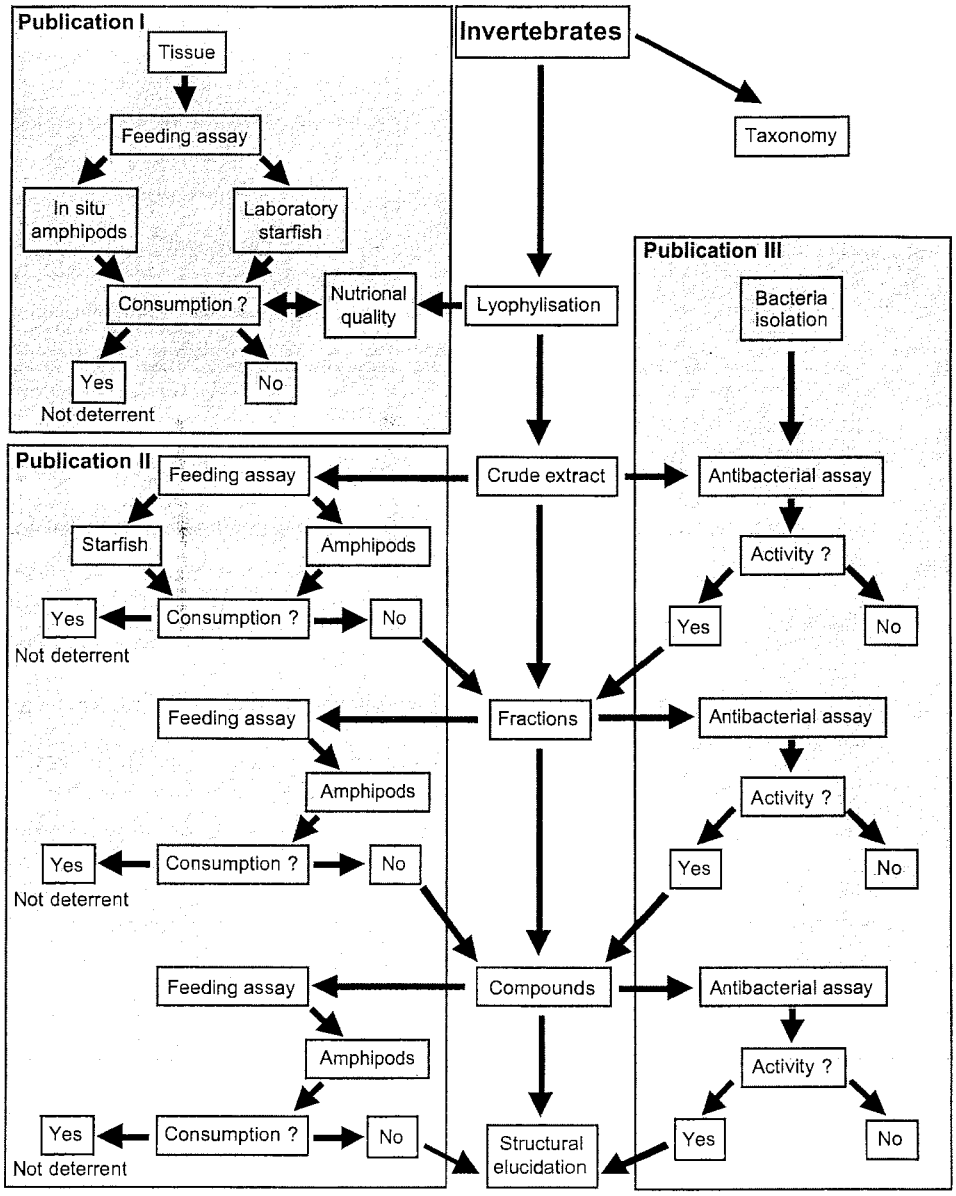


Figure 2: Flow chart of the methods used in the present study

3.1 INVESTIGATED SPECIES, SAMPLING AND SAMPLE TREATMENT

Seventeen abundant sessile or slow moving invertebrate species (Tab. 2) and the egg mass of a gastropod were collected by SCUBA diving during summer of 1999, 2000 and 2001. Samples were prepared for later processing in the following manner:

- For experiments assessing the palatability of invertebrate tissue, samples were shock frozen in liquid nitrogen and stored at –28°C until use.
- Specimens for later extraction or analysis of the biochemical composition of tissue were shock frozen, lyophilized and stored at –28°C.
- Voucher specimens were preserved in a 5% formaldehyde-seawater solution for later species identification.

3.2 BIOCHEMICAL COMPOSITION

The biochemical composition of the investigated species was analyzed by measuring protein, lipid, carbon and nitrogen content. The freeze-dried invertebrate tissue was processed according to well-established protocols. A detailed description of the methods used is given in publication I.

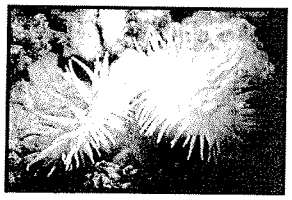
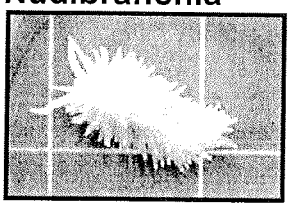
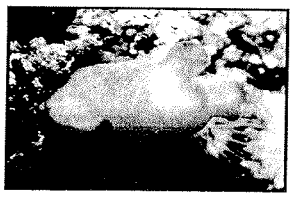
(1) Soluble protein content was determined using a commercial Bradford (Bradford 1976) protein test (Biorad). To allow comparison of data in the present study to a broad spectrum of literature, samples were extracted by two different methods giving values for NaOH-soluble and NaCl-soluble proteins.

(2) Lipid content was determined using a gravimetric technique described by Hagen (1988).

(3) Carbon and nitrogen were analysed according to the protocol of Verardo et al. (1990) using an elemental analyser for carbon and nitrogen.

(4) Water content was determined by measuring subsequently wet weight (WW) and dry weight (DW) after lyophilization.

Table 2: Investigated invertebrate species and egg mass, sampling site and depth of sampling.

	Species	Sampling site	Depth (m)
Porifera			
	*Haliclona rosea*	Hansneset	10-25
	Haliclona viscosa	Hansneset	15-25
	Spongosorites genitrix	Cave	4-5
	Suberites ficus	Cave	4-5
Cnidaria			
	*Gersemia rubiformis*	Cave	2-4
	Hormathia nodosa	Hansneset	10-25
	Urticina asiatica	Kongsfjordneset	10-25
	Urticina eques	Kongsfjordneset	10-25
Bryozoa			
	*Alcyonidium gelatinosum*	Hansneset	1-8
	Crisiella sp.	Hansneset	1-10
	Eucratea loricata	Hansneset	1-6
	Tricellaria ternata	Hansneset	1-10
Nudibranchia			
	*Dendronotus frondosus*	Different sites	1-25
	Flabellina salmonacea	Different sites	1-25
Ascidiacea			
	*Halocynthia pyriformis*	Hansneset	5-25
	Styela spp. (*S. rustica, S. gelatinosa*)	Prince Heinrich Islands	15-30
	Synoicum turgens	Hansneset	1-7

3.3 PALATABILITY OF INVERTEBRATE TISSUE

3.3.1 *In situ* experiments

To determine if invertebrate tissue would be consumed by naturally occurring consumers, ten of the investigated species were assayed for their palatability *in situ*. Fish, serving as a control food, and test tissue from one invertebrate species were offered at the same time. Test and control tissue were cut into 1 cm^3 cubes and attached to buoyant ropes using labeled safety pins. For any experiment, a total of 18 test food pieces per species and 18 control pieces of fish were distributed randomly among 12 ropes. These ropes were placed at 4 - 5 m depth in the Kongsfjord by tying them to metal bars. Water temperatures did not allow continuous observation of the experiment but regular checks were performed to describe active consumers. After 24 hours numbers of pieces of each food type remaining attached to the rope were counted and difference in consumption between test and control food was compared by Fisher's exact test (Sokal & Rohlf 1981).

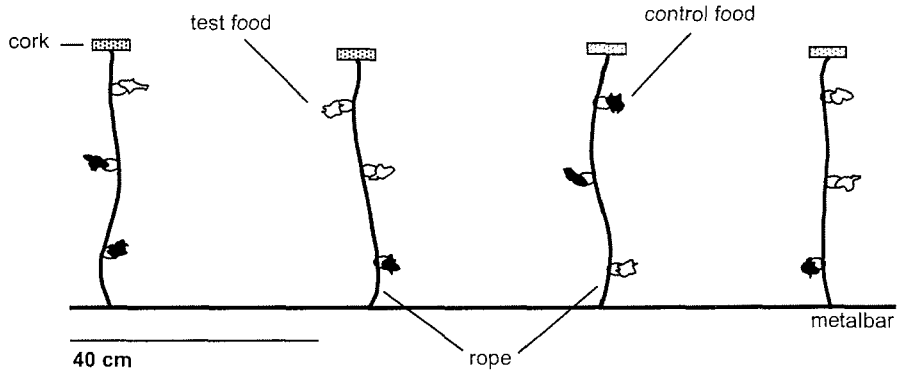


Figure 3: Principle design of experimental set up of *in situ* assays in the Kongsfjord

3.3.2 Laboratory assay with starfish

To further assess the palatability of invertebrate tissue under controlled conditions, laboratory experiments with the starfish *Asterias rubens* from the North Sea were performed. Specimens of *Asterias rubens* were collected around the island of Helgoland (North Sea) by SCUBA diving and maintained in laboratory aquaria at the Alfred Wegener Institute, Bremerhaven. Sympatric predatory starfish from

Kongsfjord could not be maintained under laboratory conditions in sufficient numbers, therefore *Asterias rubens*, a close relative of the Arctic predatory species *Asterias amurensis*, was used as a model organism in this study. Starfish were always starved for two days prior each feeding experiment. During the assay animals were kept individually in plastic bowls filled with seawater. One piece of invertebrate tissue to be tested for palatability (hereafter referred to as "test tissue") was offered to each of 20 starfish by placing the animal on top of the bite-sized food item. An equal number of starfish was offered a piece of "control tissue" (fish) at the same time. A food item was considered rejected as soon as a starfish moved away from the food. After each starfish had either rejected or consumed the respective food item but at least after eight hours, significant differences in consumption between test and control food were determined by Fisher's exact test (Sokal & Rohlf 1981).

3.4 EXTRACTION PROCEDURE, FRACTIONATION AND ISOLATION

To obtain crude extracts a known mass of freeze-dried tissue from each species was extracted in methanol:dichloromethane (1:1). The yield of organic crude extract per g DW or per g WW of invertebrate tissue (Table 1, publication II and III) is referred to as the natural concentration and was used to calculate the amount of extract employed in the experiments investigating chemical defenses (see below). Extracts of those species that showed antifeeding and/or antibacterial activity were subsequently fractionated using solvents of different polarity. For one species, the sponge *Haliclona viscosa*, two active fractions were further purified, using low-pressure liquid chromatography and preparative reversed-phase high-pressure liquid chromatography.

3.5 CHEMICAL DEFENSE AGAINST PREDATION

In a second set of experiments crude extracts, fractions and pure compounds of the invertebrates were incorporated into artificial food and tested for potential feeding deterrent effects. Two general consumers with different feeding modes

were employed in these assays, again the starfish *Asterias rubens* and the abundant amphipod *Anonyx nugax* from the Kongsfjord.

3.5.1 Preparation of artificial food

Artificial food pellets were prepared with alginic acid using freeze-dried and finely powdered fish as a feeding stimulant. Crude extracts, fractions or isolated compounds were added in a small volume of solvent at natural concentrations to the powdered fish and then the solvent was removed from the mixture by rotary evaporation. For control food the fish was treated with an equivalent amount of solvent only. Pellets containing organic crude extracts, fractions or compounds are hereafter referred to as "test pellets". Pellets without any extract are referred to as "control pellets".

3.5.2 Assay with starfish

Assays with *Asterias rubens* to assess the palatability of crude extracts were conducted in a similar manner as those testing invertebrate tissue. The only difference was that instead of offering test and control tissue to different individuals at the same time (see section 3.4.2), the same individuals were offered test and control pellets subsequently. A control pellet was offered to the starfish first. If the control pellet was consumed, then the same starfish was offered a test pellet. If the test pellet was rejected, the starfish was offered a second control pellet to determine whether the test pellet was rejected because the starfish was satiated. If a starfish consumed a control pellet after rejecting a test pellet, it was concluded that the test pellet was rejected due to the added crude extract, fraction or compound. Only those replicates were included in the analysis in which both the first and the second control pellet were eaten. The experiment was analysed using a Fisher's exact test (Sokal & Rohlf 1981).

3.5.3 Assay with amphipods

Numerous individuals of the abundant amphipod *Anonyx nugax* were caught in the Kongsfjord using traps baited with fish and kept in laboratory aquaria at Ny Ålesund research station. For feeding experiments, in each of eight containers 20 amphipods were placed. Each container held one test pellet and one control pellet.

To determine changes in pellet mass caused by water uptake or loss of material during the experiment due to container effects, an equal number of beakers without amphipods contained also one test and one control pellet (control containers). All pellets were gently blotted and weighed before the experiments. Amphipods were removed when about half of the pellets in the experimental containers had been consumed, but latest after 8 hours. After the experiment, all test and control pellets were blotted again and weighed to determine mass changes during the experiments. Data were analysed according to Peterson & Renaud (1989) (for details see publication II).

3.6 ANTIBACTERIAL EFFECTS OF SECONDARY METABOLITES

3.6.1 Bacterial isolation and sequence analysis

Bacterial strains were isolated from stones, sediment and seawater in the vicinity of the investigated invertebrates. Details for sample treatment, media used and isolation procedure are given in publication III. Five bacterial strains were isolated and taxonomically identified by sequence analysis of 16S rDNA.

3.6.2 Antimicrobial assay

To identify potential antibacterial activity of the investigated invertebrates against the five sympatric bacterial strains the agar disc-diffusion assay (Acar 1980) was used (publication III). Crude extracts and fractions were dissolved in aliquots of the extraction solvent to give natural concentrations (Table 1, publication III). Crude extracts, fractions or pure compounds were applied onto each side of a sterile paper disc and the solvents were then allowed to evaporate. Control discs were prepared in the same manner with solvent only. Extract discs and solvent control discs were placed on the surface of agar plates previously seeded with individual bacterial strains. The radius of the inhibition zone (without disc) was measured to the nearest 0.5 millimeter following incubation at 4°C over 5 days, and used as indicator for the degree of antimicrobial activity.

4 RESULTS

In the following the results are summarized and it is referred to Figures and Tables given in the three publications.

4.1 PALATABILITY OF INVERTEBRATE TISSUE

In situ assays for palatability were performed with ten invertebrate species (three sponges, two actinians, one octocoral, one bryozoan, three ascidians) exposing tissue to natural consumer assemblages. From diving observations during the experiments it is suggested that these assemblages mainly consisted of amphipods. With the exception of the ascidian *Styela* spp., all invertebrates were consumed significantly less compared to the control food offered simultaneously (publication I, Fig. 2).

Results of palatability assays of sixteen invertebrate species against the predatory starfish *Asterias rubens* are shown in publication I, Fig. 3. These experiments were carried out under controlled laboratory conditions, and were very similar to the results obtained from the field assays. From those species tested in field and laboratory assays, the ascidian *Halocynthia pyriformis* and the bryozoan *Alcyonidium gelatinosum* were rejected by the natural predator assemblage but did not significantly deter starfish feeding. In total, four species were not significantly rejected by the starfish while the other twelve investigated invertebrate species significantly inhibited starfish feeding.

- 90% of species tested *in situ* were significantly rejected by natural assemblages of consumers.
- Invertebrate tissue of 75% of the species tested significantly inhibited starfish feeding in the laboratory.

4.2. BIOCHEMICAL COMPOSITION OF INVERTEBRATES

The biochemical composition of the investigated invertebrates, expressed as water content, lipid content, NaOH- and NaCl-soluble protein, nitrogen content, and as carbon to nitrogen ratio is given in publication I, Table 2. Considering all measured parameters, the actinian *Urticina* aff. *eques* and the sponge *Suberites ficus* showed highest values, followed by the nudibranchs *Dendronotus frondosus* and *Flabellina salmonacea* and the actinian *Hormathia nodosa*. Species with relatively low biochemical parameters were the egg mass of *Natica* sp., the bryozoans *Tricellaria ternata* and *Eucratea loricata,* the sponge *Spongosorites genitrix* and the ascidian *Synoicum turgens*.

- The biochemical composition of the invertebrates studied exhibited high variation between the different species.

4.3 FEEDING DETERRENCE OF SECONDARY METABOLITES

Results of palatability assays with *Anonyx nugax*, testing crude extracts of seventeen invertebrate species and the gastropod egg mass are given in publication II, Fig. 2. The extracts of the sponge *Haliclona viscosa* and the actinian *Hormathia nodosa* significantly deterred amphipod feeding, whereas the addition of crude extracts from six other species, had a significantly stimulating effect on amphipod feeding. Crude extracts of the remaining ten species had no significant effect on the consumption by amphipods at natural concentrations. The crude extract of the soft coral *Gersemia rubiformis* had an inhibiting effect on amphipod feeding at concentrations five-fold higher than natural (publication II, Fig. 4).

Sixteen of the invertebrate species investigated in the amphipod assay were also tested in the feeding experiments with the starfish *Asterias rubens* from the North Sea (publication II, Fig. 3). Overall, the results of the starfish assay are very similar to those of the assays with amphipods.

Extracts of the sponge *Haliclona viscosa* and the actinian *Hormathia nodosa*, both of which were significantly rejected at natural concentrations by at least one of the predators, were further purified, and fractions of both species as well as pure compounds from *H. viscosa* were tested in the amphipod assays. The *n*-

BuOH or EtOAc/*n*-BuOH soluble compounds from both species and the *n*-hexane soluble compounds from *H. viscosa* were significantly deterrent to *Anonyx nugax* (publication II, Fig. 5 and 6). In experiments with compounds isolated from *H. viscosa,* one compound of the *n*-hexane soluble and one compound of the *n*-BuOH soluble fraction showed feeding deterrent activity.

- From the crude extracts tested against amphipods 11% deterred feeding while 33% had a stimulating effect.
- Out of 16 crude extracts tested against *Asterias rubens* only one was significantly rejected.
- One fraction of *Hormathia nodosa* and two fractions of *Haliclona viscosa* were bioactive.

4.4 ANTIBACTERIAL ACTIVITY

Bacterial isolates from the vicinity of the investigated invertebrates are phylogenetically diverse and clustered within clades of previously cultured bacteria from polar oceans or sea ice (publication III, Fig. 2). Results of antimicrobial experiments testing crude extracts of the investigated invertebrate species against sympatric bacteria are shown in publication III, Table 3. Antibacterial effects differed with respect to extracts and bacterial strains. Six out of 18 extracts inhibited growth of at least one bacterial strain at natural extract concentrations. The extract of the sponge *Haliclona viscosa* had the strongest antimicrobial activity in terms of the number of strains inhibited and the radius of the inhibition zones. Further purification showed that the EtOAc/*n*-BuOH soluble compounds from *H. viscosa* and the EtOAc fraction from *H. pyriformis* inhibited bacterial growth (publication III, Table 4 and 5). The activity of *H. viscosa* was traced back to three pure compounds from the EtOAc/*n*-BuOH fraction but none of the effects caused by the individual compounds corresponded exactly with the strength of antibacterial effects caused by the crude extract (publication III, Table 4).

- 33% of invertebrates studied exhibited antimicrobial activity.
- Three pure compounds isolated from *Haliclona viscosa* inhibited bacterial growth.

5 GENERAL DISCUSSION AND CONCLUSIONS

5.1 ECOLOGICAL RELEVANCE OF FEEDING ASSAYS

Various feeding experiments were used in this study to assess palatability of invertebrate tissue and their organic extracts against predators. The experimental approach employed in the *in situ* experiments has been widely used in tropical environments (Harvell et al. 1988; Van Alstyne & Paul 1992; Schupp et al. 1999), but this was the first attempt to test prey palatability in the field in a polar region. The most abundant amphipod species captured in Kongsfjord using baited traps were the lysianassids *Anonyx nugax* and *Onisimus edwardsi,* indicating that these taxa were also the main groups feeding on the experimental foods. Many lysianassid species are known for their multiple feeding strategies, including scavenging and predation on benthic and planktonic organisms (Slattery & Oliver 1986; Legezynska 2001). This broad trophic spectrum can be viewed as an adaptation to food-limited environments, and, therefore, lysianassid amphipods are likely to be good indicators for palatability of invertebrate species in the field assays. For quantification, the field assays were compared with laboratory feeding assays. *Anonyx nugax*, the major predator in the field studies, was not a suitable organism for the laboratory assays testing invertebrate tissue, because their feeding efficiency is likely to vary with size, shape and texture of the tissue and will probably confound quantitative measures. The amphipod species, however, was used in laboratory assays to test feeding deterrent properties of extracts of invertebrates, because extracts are incorporated into food pellets of identical size, shape and texture.

Since consumers have been shown to differ in their sensitivity to feeding defenses (Pennings et al. 1994), experiments with a second consumer type, exhibiting a different feeding mode than amphipods, were performed. Starfish are common invertebrate predators and feed by extruding the stomach over a whole food item rather than biting small pieces. Carnivorous starfish, *Solaster endeca*

and *Crossaster papposus*, could not be caught and kept in sufficient numbers under laboratory conditions in Kongsfjord. Therefore, the starfish *Asterias rubens,* abundant along the entire European Atlantic up to northern Norway and a close relative of the Arctic predatory species *Asterias amurensis*, was chosen as a model organism for feeding assays. *Asterias rubens* is a generalist predator, known to feed on a large variety of macrofauna (Gulliksen & Skaeveland 1973; Anger et al. 1977). The results of feeding experiments testing tissue and extract palatability in amphipod and in starfish assays were very similar, despite the fact that *A. rubens* is not a sympatric predator and has a different feeding method than amphipods. These similar preference / avoidance reactions of both predators in relation to the invertebrate species offered indicate that, in this case, causes for prey selection seem to be of more general nature and are beyond local adaptations.

Bioassays testing chemical defense properties of invertebrate extracts were performed with extracts at natural concentrations to provide ecologically relevant dosages. However, bulk estimations from extract yield per gram dry weight extracted tissue may underestimate natural extract concentrations. Defensive substances are often not distributed evenly throughout the body of an organism but instead are concentrated in tissues that, e.g., have a higher probability to be attacked (Schupp et al. 1999), or are most valuable like reproductive regions (Pawlik et al. 1988). Considering that secondary metabolite concentrations may be higher in specific tissues than were estimated for the whole body, feeding experiments with higher extract concentrations of several conspicuous species were repeated. Only the extract of the octocoral *Gersemia rubiformis* significantly deterred amphipod feeding at higher extract concentrations while for other extracts the increase in concentration did not show any effect. The use of natural concentrations is obviously a good approach for a first screening of chemical defenses in sub-Arctic marine invertebrates.

- *Anonyx nugax* and *Asterias rubens* are suitable test organisms in feeding assays with invertebrates from the Kongsfjord.
- Natural extract concentration as estimated in this study provided reliable results about bioactivity

5.2 PREDATORS IN THE KONGSFJORD

As stated by Thorson (1957), predation is low in the Arctic and may thus not be a strong selective force to drive the evolution of chemical defenses in marine organisms from high northern latitudes. Although the effect of predators can be high in infaunal communities (Ambrose 1984, 1986), the predator prey relationships within the less abundant hard bottom communities are relatively unstudied in Arctic waters. The *in situ* feeding assays in the present investigation were performed to examine and quantify the feeding response of a natural assemblage of predators and scavengers towards tissues of benthic invertebrates, but they also may give limited insight into predation intensity in the study area. Regular feeding on control food (29-72% consumption) in the *in situ* feeding assays documented the presence of predators. However, controls were never eaten entirely. This could indicate that natural assemblages of consumers had been saturated after a limited feeding period, probably due to their generally low abundance Regular checks of the experiments suggest that mostly amphipods were feeding on the offered food. It is unlikely that fish species were feeding on the experimental food since there have been only very few observations of fishes during over 200 SCUBA dives in the Kongsfjord. However, fish cannot be excluded completely as consumers in the *in situ* experiments, because some fish species are reported from the shallow water (< 30 m) of Kongsfjord with mainly pleuronectids feeding on epibenthic fauna (Hop et al. 2002). According to Hop et al. (2002) and personal underwater observations starfish are the most conspicuous carnivorous invertebrates in Kongsfjord beside prosobranch snails (e.g. *Buccinum* sp.). While carnivorous starfish like *Solaster endeca* and *Crossaster papposus* are common in the outer Kongsfjord, mostly suspension feeding *Henricia sanguinolenta* and detritivore *Poraniomorpha hispida* were found in the inner fjord. Overall, down to 30 m water depth predation seem to play a minor role, at least in the inner part of the fjord.

- In the shallow water of Kongsfjord only low diversity and abundance of predators was observed, mainly amphipods, as well as some starfish and gastropods

  ⇒ Selective pressure seems to be low.

5.3 DEFENSES AGAINST PREDATION

As discussed above, predator abundance seems to be generally relatively low in the middle and inner Kongsfjord. In the *in situ* assays, however, significantly less consumption on most of the invertebrate tissues (0-28%) was found compared to the control food (30-70%; Fig. 2, publication I). These results clearly show that some predators are present in the Kongsfjord, and that they are able to discriminate between the different food sources. Nine out of ten invertebrate species were rejected significantly by naturally occurring predators, indicating that 90% of the species possess a protective mechanism against predation. Also, 75% invertebrate species tested in the laboratory assay deterred feeding by the starfish *Asterias rubens*. Both data sets indicate that many species from Arctic regions are protected against predators.

According to the optimal foraging theory (Hughes 1980; Pyke 1984) it would be predicted that species with higher nutritional value should be preferred prey compared to prey with lower nutritional values. Although invertebrates from the Kongsfjord seem to have relatively low nutritional values compared to invertebrates from other geographic regions (see discussion publication I), different levels of nutritional quality can be distinguished among the taxa measured in the present study. The investigated species can be grouped into three categories, high, medium and low, according to their overall nutritional quality, based on the biochemical parameters proteins, nitrogen and lipids (Table 2, publication I). If nutritional quality of prey is a key factor in food selection of predators it would be expected to see differences between feeding rates of natural assemblages as well as of the starfish *Asterias rubens* on high, medium and low nutritional quality invertebrates. However, there was no consistent positive correlation between the rejection or consumption of invertebrate tissue in the feeding assays and the nutritional quality. Therefore, it can be concluded that for most of the species tested the predictions of the optimal foraging theory are not met. It cannot be excluded that low or high nutritional quality of some invertebrates may be the reason for rejection or consumption, respectively, by the predators employed in the assays (for a more detailed discussion see publication I). It is likely, however, that in most cases other mechanisms than nutritional quality are affecting palatability of the invertebrate species tested. This is in accordance to the

findings of McClintock (1987) and McClintock et al. (1992) for Antarctic sponges and nudibranchs, respectively, who also could not find any correlation between nutritional quality of prey and predation rates. Similar results were obtained for a number of carnivores and herbivores from different geographic regions measuring various parameters for nutritional value of the prey (Carefoot 1973; Larson et al. 1980; Chanas & Pawlik 1995; Rogers et al. 1995; Stachowicz & Lindquist 2000; Granado & Caballero 2001).

- Invertebrates from the Kongsfjord are protected against predation.
- No obvious correlation between consumption and nutritional quality was found.

5.4 CHEMICAL DEFENSES

If nutritional quality is not a common way of the investigated invertebrates to be protected against predation, other defensive strategies, such as chemical or structural defenses, must be responsible for the low palatability of invertebrate tissue. Structural defenses have not been investigated in the present study, but some species might be defended by structural elements. The bryozoans *Eucratea loricata* and *Tricellaria ternata*, as well as the egg mass of *Natica* sp. for example were significantly rejected by predators most probably due to strong calcification which may act as a structural defense (Harvell 1984). In contrast, the bryozoan *Alcyonidium gelatinosum*, which was not significantly rejected by the starfish, is less calcified. Similarly, the tunic of the ascidian *Synoicum turgens* mainly consists of cellulose-like polysaccharides, which are largely indigestible to animal predators and therefore may have caused the rejection by amphipods and starfish.

When testing organic crude extracts of the investigated invertebrate species, chemical defense was only found for three out of eighteen species tested. Crude extracts of the actinian *Hormathia nodosa* and the sponge *Haliclona viscosa* were feeding deterrent at their natural concentrations against general consumers. The extract of the octocoral *Gersemia rubiformis* had slight activity, but only at higher extract concentrations. This indicates that secondary metabolites only play a minor role in protecting the investigated species from predation (Fig. 2, 3 and 4, publication II). Additionally to feeding deterrence, the crude extracts of the sponge

Haliclona viscosa and the ascidian *Halocynthia pyriformis* also inhibited the growth of sympatric bacteria. The crude extract of *H. viscosa* exhibited the strongest inhibition of bacteria and affected more bacterial strains than any other extract tested (Tab. 4; publication III). This indicates that some of the bioactive compounds have a larger spectrum of activity, including antipredator and antifouling properties.

Among the active crude extracts, the *n*-BuOH fraction of *H. nodosa* had a significant inhibiting effect on amphipod feeding, suggesting the involvement of polar compounds in feeding deterrence. In *H. pyriformis* the less polar EtOHc fraction suppressed microbial growth. So far, active metabolites have only been isolated for the sponge *H. viscosa*, and following this species will be discussed in more detail.

Haliclona viscosa

Two different fractions of the crude extract of *Haliclona viscosa* significantly inhibited amphipod feeding. This deterrence could be traced back to one compound from the *n*-hexane (K2) and one compound from the EtOAc/*n*-BuOH fraction (X2) (Fig. 6; publication II). The different polarity shows that two chemically distinct substances exhibit feeding deterrence independently. The bioactivity of several different compounds characterises *H. viscosa* as a chemically rich species compared to other invertebrates from Kongsfjord. One of the two fractions active in the feeding assays also exhibited antimicrobial activity (EtOAc/*n*-BuOH fraction) (Tab. 4; publication III). This suggests that the active *n*-hexane soluble compound specifically effects feeding of predators, while the active compound from the EtOAc/*n*-BuOH fraction has various ecological roles. Secondary metabolites with multiple functions may represent an optimization of defense, since synthesizing and maintaining chemical defenses is supposed to be energetically costly (Rhoades 1979).

As described above, the *n*-BuOH/EtOAc fraction of *H. viscosa* caused strong antimicrobial activity against a variety of different sympatric bacterial strains. Three major compounds (X2, X3, X4) were purified from this fraction, but none of these compounds alone repeated the effect of the *n*-BuOH/EtOAc fraction. Compound X2, the metabolite that also deterred feeding of sympatric amphipods, had the strongest activity and was most comparable to the effects of the crude extract. The

various combinations of compounds (Tab. 4, publication III) did not reveal any additive or synergistic effects. Newbold et al. (1999) also found inhibition zones produced by crude extracts to be greater than those of purified compounds, and suggested that the crude extract may contain additional minor bioactive agents that were not isolated. Furthermore, extraction procedures may lead to a certain compound degradation and loss of compound mass.

Several species of the genus *Haliclona* from tropical and Antarctic regions contain biologically active compounds (e.g. McClintock 1987; Baker et al. 1988; Fusetani et al. 1989; Bakus et al. 1994; Jaspars et al. 1994; Charan et al. 1996; Clark et al. 1998; Harrison 1999; Brown et al. 2001). Many *Haliclona* species are known to be especially rich in sterols (Findlay & Patil 1985; Seldes et al. 1985 a, b; Rovirosa et al. 1990; Parameswaran et al. 1994; Elenkov et al. 1999), but also terpenes and alkaloids (e.g. Parameswaran et al. 1998; Harrison 1999; Rashid et al. 2001). The Antarctic species *Haliclona dancoi* exhibited feeding deterrent activity against predatory starfish (McClintock 1987), and extracts of *Haliclona cinerea* from California and of *Haliclona mediterranea* from the Mediterranean Sea were highly active against bacteria (Thompson et al. 1985; Amade et al. 1987). However, on the other hand a few *Haliclona* species also have been shown to be inactive or only weakly active against microorganisms (*Haliclona heterofibrosa*, Bergquist & Bedford 1978; *Haliclona* sp., McClintock & Gauthier 1992). Interestingly enough, of the two species of the genus *Haliclona* (*H. viscosa*, *H. rosea*) occurring in the sub-Arctic Kongsfjord, only the extract of *H. viscosa* showed feeding deterrent and antibacterial activity. Since both species are exposed to very similar environmental and biological conditions, this could suggest that these factors are less important than species-specific traits.

Sponges of the order Haplosclerida (class Demospongiae, subclass Ceractionmorpha) are generally known to often contain various 3-alkylpyridines or their reduction products, especially the genera *Haliclona*, *Xestospongia* and *Amphimedon*. These are structurally diverse but related, polycyclic alkaloids with two heterocyclic nitrogens and no aliphatic methyl groups. Several of these compounds are known to have strong cytotoxic activity (Andersen et al. 1996). The structural elucidation of the three substances isolated from *H. viscosa* in the present study showed that X2 is related to the alkaloid cyclostelletamine C described from *Stelletta maxima* (Fusetani et al. 1994), while X3 and X4 are

related to the alkaloid haliclamine A, from *Haliclona* sp. (Fusetani et al. 1989). These species were collected in Japan. Both X3 and X4 from *H. viscosa* consist of macrocyclic bis-tetrahydropyridines linked through two alkyl chains. While X3 exhibits C_9 and C_{11} alkyl chains, X4 shows a C_{10} chain instead of C_9 resulting in different molecular formula and mass (Fig. 3).

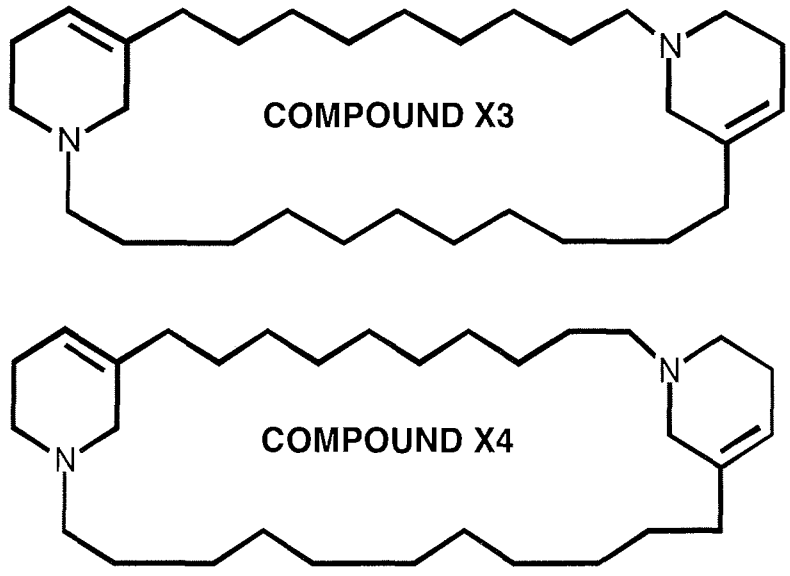


Figure 3: Chemical structures of the secondary metabolites X3 and X4 isolated from the sponge *Haliclona viscosa* (working group of Dr. Köck. Alfred Wegener Institute for Polar and Marine Research, Bremerhaven).

Although the chemistry of the secondary metabolites in *H. viscosa* from the Kongsfjord has been recently documented (working group of Dr. Köck, Alfred Wegener Institute for Polar and Marine Research, Bremerhaven), nothing is known so far on the underlying molecular mechanisms of bioactivity. Laboratory observations revealed extremely high mortality of amphipods when these animals were held together with *Haliclona viscosa* in the same container for several hours, indicating the presence of highly toxic compounds.

- Only few species are protected by chemical defense.
  ⇒ Structural defense may play an important role.
- Of the two species of the genus *Haliclona* occurring in the Kongsfjord only *H. viscosa* exhibited biological activity.
- *Haliclona viscosa* is a chemically rich species.
- EtOAc/n-BuOH soluble compounds of *H. viscosa* have different ecological functions (feeding deterrence, antimicrobial activity).
- *H. viscosa* has broad-spectrum antibacterial compounds.
- The underlying chemical structures are closely related to known alkaloids.

5.5 LATITUDINAL COMPARISON

Bakus & Green (1974) suggested an inverse relationship between the incidence of chemical defense in marine invertebrates and latitude, due to a lower selective pressure of predation in higher latitudes. For Antarctic regions, however, several recent studies have shown that this hypothesis does not hold true (Amsler et al. 2000; Avila et al. 2000). In Antarctic waters, predation by invertebrates has been found to exert selective pressure for the development of chemical defenses (McClintock & Baker 1997). The latitudinal hypothesis, however, has not been tested yet towards the northern extension of the latitudinal gradient, and virtually nothing is known on the occurrence of chemical defenses in Arctic waters. The present study is one of the first to address the topic of the abundance and ecological significance of bioactive secondary metabolites in invertebrates from Northern high latitudes. The incidence of feeding deterrence found in invertebrates from Kongsfjord is very low. Only three out of eighteen species (16%) exhibited chemical defense against predation. Similarly, only a small portion (33% of 18 species tested) of invertebrates is chemically defended against bacteria compared to species from lower latitudes and also from Antarctica. For example, of 32 Caribbean gorgonians all were feeding deterrent (O'Neal & Pawlik 2002), and 69 % of 71 Caribbean sponges yielded deterrent extracts (Pawlik et al. 1995). McClintock & Gauthier (1992) found 64% of 17 Antarctic sponges to be at least weakly active against marine bacteria. In New Zealand, 76% of 30 sponges inhibited growth of marine bacteria (Bergquist & Bedford 1978), 48% of 33

Caribbean sponges had antimicrobial activity (Newbold et al. 1999), and 32% of 28 Mediterranean sponges tested against marine bacteria and yeasts were active (Amade et al. 1987). Thus, the results from antifeeding and antibacterial experiments in the present study indicate a low percentage of chemically defended invertebrates compared to tropical and Antarctic regions, and it is also in the lower range of that found in temperate waters (*fide* Bakus & Green 1974). Therefore, the results from Kongsfjord confirm the inverse relationship between latitude and incidence of chemical defense according to the hypothesis of Bakus & Green (1974).

Although the different frequency of chemical defenses against predation between Arctic and Antarctic regions might be explained by differences in selective pressure, i.e. lower abundance of predators in Kongsfjord, the question remains why also a lower incidence of antimicrobial activity has been found in invertebrates from the Kongsfjord. Cold adapted microorganisms have been described in similar presence and abundance from Antarctic and Arctic waters (Zajaczkowska & Zajaczkowski 1989; Zdanowski 1995; Knoblauch 1999), and there is no evidence to assume strong differences in bacterial pressure between both polar regions. Comparisons of the number of bacterial strains that could be cultured from invertebrate surfaces showed that bacteria colonized the investigated species to different degrees, although this is by no means a quantitative measure of the actual degree of microbial fouling (publication III). These observations suggest that there may be mechanisms other than secondary metabolites responsible for antifouling properties of some of the invertebrates from Kongsfjord, for example, mechanical or physical defenses like tissue sloughing (Barthel & Wolfrath 1989), mucus secretion (Krupp 1985) or surface acidity (Hirose et al. 2001).

Kongsfjord, although located at high latitude, is under the influence of the relatively warm West Spitsbergen Current carrying water from the northernmost extension of the North Atlantic Current (Svendsen et al. 2002), and has to be regarded as a sub-Arctic rather than a high Arctic fjord (Hop et al. 2002). Rozycki (1990) classified the western Spitsbergen area as the transition zone between arctic and boreal regions based on its faunal composition. But even more importantly, Kongsfjord is strongly influenced by glacial activity. High sedimentation rates, freshwater influence and ice scouring are known to have a

considerable structuring effect on the fauna especially of inner fjord locations (Gulliksen et al. 1985; Wlodarska-Kowalczuk et al. 1998). Gulliksen et al. (1985) recorded the highest number of species in transects outside Van Mijenfjord and Raudfjord, with the number of species and diversity decreasing towards the inner part of both fjords. Therefore, biological dynamics in Kongsfjord might be representative for western Spitsbergen fjord environments (Hop et al. 2002), but conditions may be very different at other locations in high northern latitudes that are more under the influence of Arctic water masses, e.g. the northern and the eastern shores of Spitsbergen (Wlodarska-Kowalczuk et al. 1998). Although the present findings with respect to the incidence of chemical defenses in Kongsfjord support the latitudinal hypothesis, it has to be kept in mind that this is a single location and may not be representative for other Arctic locations. More chemical ecology studies at different sites in the high northern hemisphere are necessary to either reject or support the latitudinal hypothesis in general for Arctic waters.

- The latitudinal hypothesis is supported by the results presented.
- Due to the influence of Atlantic water masses Kongsfjord might not be representative for high Arctic locations

5.6 THE ARCTIC AS A SOURCE FOR NEW MARINE NATURAL PRODUCTS

The results from the present study indicate a low incidence of bioactive compounds in marine invertebrates from the Kongsfjord. In addition, species richness and endemism in the Arctic are low compared to tropical regions and the Antarctic (Dunton 1992; Grebmeier & Barry 1991). Furthermore the logistic requirements for routine collection of invertebrates in the Arctic are much higher than at more temperate sites. Considering all these factors the probability to discover new marine natural products for applied purposes can be expected to be low. Additionally, many invertebrates occur in low biomass, especially species of the less abundant hard bottom fauna. Therefore only few taxa can be collected in sufficient amounts for commercial purposes without the risk of overexploiting these biological resources.

5.7 FUTURE PERSPECTIVES

- The present study found evidence that the incidence of chemical defense is low in Arctic waters. However, conditions at other Arctic locations, especially in the high Arctic, may be very different compared to those in western Spitsbergen fjords. Therefore, more chemical ecology studies at different sites in the high northern hemisphere are needed to assess the incidence of chemical defense in Arctic waters.
- Most of the investigated species in the present study also occur at lower latitudes. *Haliclona viscosa*, *Haliclona rosea*, *Suberites ficus*, *Alcyonidium gelatinosum* and *Dendronotus frondosus*, for example, also occur in the North Sea where they are probably exposed to a higher selective pressure through predation. In the context of evolutionary questions about bioactive natural compounds and the adaptive influence of abiotic and biotic factors versus genetically fixed traits, the assessment of defensive strategies in different populations under different environmental conditions could enhance the present knowledge. For example, the sponge *Suberites ficus* from Kongsfjord did not show antimicrobial activity, but extracts of the same species from southern Britain were active against a marine and a non-marine bacterial strain (Dyrynda 1985). Direct comparisons between these different studies, however, have to be considered cautiously because of the variety of methods used and the different bacterial strains employed.
- Variability in secondary metabolite concentration can be high between different tissues of the same species. Further studies should address this issue in taxa that were found to be chemically defended.
- The level of predation in the Kongsfjord is still largely unknown, especially in a seasonal context. A detailed analysis of the trophic web in this fjord, e.g., through gut content analysis and stable isotope analysis, could reveal important information about the level of predation and would allow conclusions on the impact on the development of chemical defenses in invertebrates.
- Similar antimicrobial and antifeeding activity was found in taxonomically closely related octocorals from both polar regions. The extract of *Gersemia antarctica*, a close relative to *Gersemia rubiformis* tested in this study, showed growth

inhibition of sympatric bacteria similar to the present results (Slattery et al. 1995). Both, *G. antarctica* and *G. rubiformis*, contain also bioactive compounds which deter feeding of sympatric predators (Slattery & McClintock 1995; present study). In terms of evolutionary questions it would be interesting to investigate if the bioactive compounds are chemically identical in both species.

- Although some chemical structures of bioactive compounds isolated from the investigated invertebrates have been identified, other structures have still to be elucidated.
- A general problem in chemical ecology is the lack of knowledge on the underlying molecular mechanism of biological function of secondary metabolites. Special molecular and biochemical techniques need to be developed to address this important issue.

6 PUBLICATIONS

This thesis consists of the three publications listed below. The contribution of each author is outlined.

PUBLICATION I

H. Lippert and K. Iken
Palatability and nutritional quality of marine invertebrates in a sub-Arctic fjord

The two authors discussed the conceptual frame of this paper and developed the experimental design. The first author carried out the experiments and conducted the data analysis and the interpretation of results. The first version of the manuscript was written by the first author. The second author discussed this draft with the first author and improved the manuscript.

Submitted to Journal of the Marine Biological Association of the United Kingdom

PUBLICATION II

H. Lippert, K. Iken, C. Volk, M. Köck and E. Rachor
Chemical defense against predators in a sub-Arctic fjord

The first and the second author developed the initial idea of this paper. The experimental design was worked out by the first author and discussed with the second author. The first author conducted the field work, extracted invertebrate tissue, performed the laboratory experiments and processed the data. Some of the crude extracts, the fractions and all of the pure compounds tested in the feeding assays were provided by the third author. The third and the fourth author worked on the structural elucidation of the pure compounds. Data were interpreted and the manuscript was written as a first version by the first author. The second author discussed this draft with the first author and improved the manuscript. The final version was achieved with the comments of the fourth and the fifth author.

Submitted to Journal of Experimental Marine Biology and Ecology

PUBLICATION III

H. Lippert, R. Brinkmeyer, T. Mülhaupt and K. Iken

Antimicrobial activity in sub-Arctic marine invertebrates

The original concept of this paper was elaborated in cooperation of the first and the fourth author. The first author developed the experimental design. Practical work was done by the first author in collaboration with the second author for sequence analysis of bacterial isolates and with the third author for preparation of crude extracts. The first author processed all data and wrote the first version of the manuscript which was revised in discussion with the second and the fourth author.

Polar Biology 2003, 26: 591-600

PUBLICATION I

PALATABILITY AND NUTRITIONAL QUALITY OF MARINE INVERTEBRATES IN A SUB-ARCTIC FJORD

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ABSTRACT

Predator-prey relationships are still little understood for Arctic and sub-Arctic hard substrate communities. Predation among invertebrates is suggested to be low, and benthic communities supposedly are structured mainly by physical factors. We propose, however, that isolated patches of hard bottom epifauna in a sub-Arctic fjord may be an important food source for some predatory organisms in a largely soft bottom dominated environment. To investigate for the first time the palatability of abundant sub-Arctic sessile or sluggish invertebrates and their value as a food source, we performed *in situ* experiments with natural consumer assemblages in the fjord. These experiments were complemented with quantitative laboratory assays, using the generalist predatory starfish *Asterias rubens* from the North Sea. Feeding preference and avoidance reactions were similar in both assays. Natural assemblages of predators *in situ* rejected nine out of ten species tested, and twelve out of sixteen species were unpalatable in laboratory assays. Results of both assays were compared to the biochemical composition of the investigated species to see whether palatability and feeding preferences coincide with nutritional quality of the prey. Nutritional quality, expressed as protein, lipid, nitrogen, carbon and water content, may account for some of the feeding preferences found, but no overall relationship between nutritional value and palatability or feeding preferences was detected.

INTRODUCTION

Kongsfjord is an open fjord under the influence of both Atlantic water as well as glacial input. Because of the Atlantic influence the fjord is to be regarded as sub-Arctic rather than Arctic, despite its location at high latitude (Hop et al., 2002). Based on zoogeographical composition western Spitsbergen is classified as a transition zone between Arctic and boreal regions (Wlodarska-Kowalczuk et al.,

1998). Large areas of Kongsfjord (below 5-10 m depth), particularly in its inner basin, are composed of poorly consolidated soft mud deposits from the outflow of adjacent glaciers (Hop et al., 2002). High sedimentation rates, ice scouring and freshwater influence lead to a depauperate epibenthic fauna in these soft sediment areas (Curtis, 1975; Elverhøi et al., 1983; Piepenburg et al., 1996; Wlodarska-Kowalczuk et al., 1998). Hard substrate is found in many intertidal areas down to around four meters depth but are mostly uncolonized by invertebrates due to ice abrasion. Larger areas of colonized hard substrate are restricted to few sites, mainly Hansneset in the middle and Kongsfjordneset in the outer part of the fjord. These locations are exposed to wave action and current and can be densely populated by sessile filter feeders. These animals may represent up to 90% of the standing biomass at depth below 15-20 m (Hop et al., 2002). Apart from the dominating barnacle *Balanus balanus*, also sponges, actinians, sedentary polychaetes, molluscs and ascidians can be abundant. Beside these restricted locations, also ice rafted drop stones, which are common throughout the fjord (Whittington et al., 1997), provide a substratum for the development of sessile hard bottom communities. Although such hard bottom communities are patchily distributed throughout the Kongsfjord, their high accumulations of invertebrates may provide an important feeding ground for epibenthic predators in a soft bottom dominated habitat. Especially soft bodied, sessile or sluggish species such as sponges, ascidians, actinians, soft corals, and nudibranchs appear to be physically vulnerable to predation and probably have little ability to actively avoid predation (Chanas & Pawlik, 1996).

Two recent reviews (Hop et al., 2002; Svendsen et al., 2002) give a comprehensive overview on the actual biological and physical knowledge of Kongsfjord and emphasize its significance as a model ecosystem for western Spitsbergen fjords. The benthic food web of the fjord, however, is still little understood (Hop et al., 2002). Although Thorson (1957) and Gulliksen (1979) found predation on benthic communities to be sparse in polar regions, locally predation by walruses (Oliver et al., 1985) or gray whales (Nerini & Oliver, 1983; Highsmith & Coyle, 1992) can have a significant impact. Among lower trophic levels predator prey relationships are relatively unstudied in Arctic waters, especially within the less abundant hard bottom communities. We hypothesize that if food sources are highly limited then even a few abundant predators may still

create strong feeding pressure. Consequently, the hard bottom community patches of high biomass and diversity could also be regarded areas of high biological activity, and biological interactions such as predation may play an important role in structuring these communities.

The aim of our investigation was to assess for the first time the palatability of conspicuous members of shallow subtidal (≤ 30 m) hard bottom communities in such a high latitude ecosystem. Field assays were performed in Kongsfjord to see whether natural predators are present and to test their prey selectivity. *In situ* assays were complemented by laboratory assays to test palatability of invertebrate tissue of different taxonomic groups.

A second objective of this study was to examine the nutritional quality of benthic invertebrates, as measured by protein, lipid, nitrogen and water content, to estimate their potential as a prey. Optimal foraging models predict that prey should be chosen to maximize the energetic benefit per foraging effort (Hughes, 1980; Pyke, 1984). For invertebrates and marine algae it has been shown that prey nutritional quality (e.g. high protein or lipid content) can strongly influence susceptibility to predators (Duffy & Paul, 1992; Penney, 2002).

MATERIAL AND METHODS

STUDY SITE

The study was carried out in Kongsfjord at the northwestern part of Spitsbergen (79° N, 12° E) (Fig. 1). The fjord is 26 km long, has a width between 3 and 8 km, and a maximum depth of 400 m. The shores consist of steep rocky bottom as well as of weakly declining slopes with mostly soft glacier sediments. The tidal range is about 2 m with weak currents (Ito & Kudoh, 1997). Four glaciers and a number of glacier run-offs add terrestrial sediments (Elverhøi et al., 1983) and freshwater to the fjord, which may reduce salinity locally from an average of 34 down to 20 PSU. The annual mean water temperature is slightly above 0° C (Ito & Kudoh, 1997), however, in summer maximum temperatures of about 6° C at the surface and of about 4° C at 20 m depth are measured (Hanelt et al., 2001).

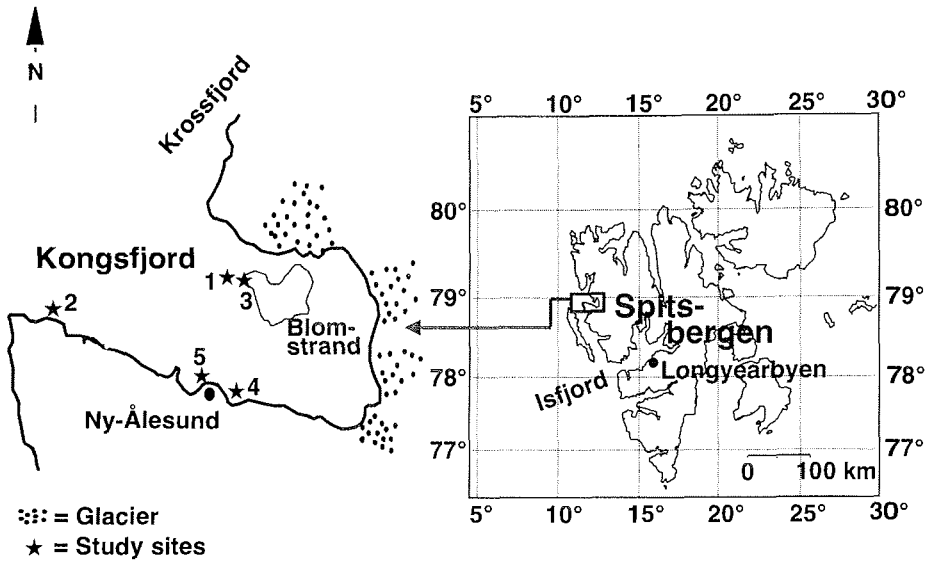

Figure 1: General view of Spitsbergen, showing Kongsfjord and the study sites. Asterisks and numbers indicate the different sites of sampling (1-4) and the site (5) where the field assays were performed: 1 – Hansneset; 2 – Kongsfjordneset; 3 – Cave, 4 – Prinz Heinrich Islands; 5 – Nansen Bay.

We collected invertebrates at four different sites in Kongsfjord. Hansneset (Site 1, Fig. 1), where most of the samples were taken, is located at the western side of the island Blomstrand in the central part of Kongsfjord. Hansneset has steeply declining rocky bottom. Site 2 (Fig. 1), Kongsfjordneset, is situated close to the entrance of the fjord. This site also consists of rocky bottom, which slopes steeply from the 15 m isobath, with occasional step-like terraces (Jørgensen & Gulliksen, 2001). The impact of ice is relatively low at both sites because there is often no sea ice formation during winter in the central and outer part of the fjord. In addition, the ice cover in these areas breaks up early in the year due to winds and currents, which in turn prevent ice from accumulating at the exposed sample locations. Hansneset and Kongsfjordneset are densely populated with rich communities of sessile invertebrates consisting of actinians, ascidians, bryozoans, sponges, bivalves and barnacles. A similarly rich but differently structured macrobenthic community is found at site 3, a cave of about 10 m in diameter close to Hansneset. Inside the cave water depth is 5 m at high tide, the walls are of solid rock while soft sediments cover the bottom. Here, octocorals and sponges prevail beside bryozoans, actinians, and colonial and solitary ascidians. Site 4, located in

the inner part of the fjord, consists of soft sediments, mostly derived from river run offs and glaciers. Single stones and boulders form patches of hard substrate. Slopes are mostly weak and impact of ice is higher compared to the other three sites. Scours of drifting ice bergs were observed down to 30 m depth. The epifaunal community is relatively poor, dominated only by one ascidian species, bryozoans and barnacles.

INVERTEBRATE SAMPLING

Table 1: Investigated invertebrate species and egg mass, sampling site and depth of sampling

	Species	Sampling site	Depth of sampling [m]
Porifera	*Haliclona rosea*	Hansneset	10-25
	Haliclona viscosa	Hansneset	15-25
	Spongosorites genitrix	Cave	4-5
	Suberites ficus	Cave	4-5
Cnidaria	*Hormathia nodosa*	Hansneset	10-25
	Urticina aff. *eques*	Kongsfjordneset	10-25
	Gersemia rubiformis	Cave	2-4
Bryozoa	*Tricellaria ternata*	Hansneset	1-10
	Eucratea loricata	Hansneset	1-6
	Alcyonidium gelatinosum	Hansneset	1-8
Gastropoda	*Natica* sp. (egg mass)	Different sites	4-30
Nudribranchia	*Dendronotus frondosus*	Different sites	1-25
	Flabellina salmonacea	Different sites	1-25
Ascidiacea	*Halocynthia pyriformis*	Hansneset	5-25
	Styela spp.	Prinz Heinrich Islands	15-30
	Synoicum turgens	Hansneset	1-7

Sixteen abundant sessile invertebrate species representative for the communities of the four sites were collected from their natural habitat by SCUBA diving and immediately brought to the laboratory. Investigated species and the according sampling sites are listed in Table 1. Later identification showed that the collection of *Styela* spp. consisted of a mixture of the two morphologically extremely similar species *Styela rustica* (LINNAEUS, 1767) and *Styela gelatinosa* TRAUSTEDT, 1886. When present, epibiontic organisms were removed from the surface of the invertebrates. Specimens for palatability assays and for analysis of their biochemical composition (see below) were shock frozen in liquid nitrogen and stored at –28°C until use. Voucher specimens were preserved in a 5% formaldehyde-seawater solution for later species identification.

IN SITU PALATABILITY ASSAYS

Ten of the species listed in Table 1 were assayed for their palatability *in situ* to determine if invertebrate tissue would be consumed by naturally occurring consumers. This experimental approach has been widely used in tropical environments (Harvell et al., 1988; Van Alstyne & Paul, 1992; Schupp et al., 1999) but to the best of our knowledge this is the first attempt to test prey palatability *in situ* in a polar region. Fish, serving as a control food, was offered at the same time as the invertebrate tissue. Test tissue from several individuals per species and the control tissue were cut into 1 cm^3 cubes and attached to 40 cm long pieces of 4 mm thick rope using labeled safety pins. Three tissue pieces per rope were attached equidistantly. For one experiment, a total of 18 test food pieces per species and 18 control pieces of fish were distributed randomly among 12 ropes. These ropes were placed in the field by tying them to metal bars and were buoyed by pieces of cork. An experiment to test palatability of one invertebrate species consisted of three metal bars (140 cm length) with four ropes each. The experiments were performed at 4 - 5 m depth (site 5, Fig. 1). Water temperatures did not allow continuous observation of the experiment but regular checks were performed to describe active consumers. After 24 hours numbers of pieces of each food type remaining were counted and difference in consumption between test and control food was compared by Fisher's exact test (Sokal & Rohlf, 1981). At the end of an experiment we thoroughly checked for tissue pieces possibly disconnected from the ropes. In the few cases where tissue cubes were found on the ground, these pieces were not included in the statistical analysis. Due to very slow currents at the experimental site it is unlikely that tissue pieces had drifted away.

LABORATORY PALATABILITY ASSAY

To further assess the palatability of those invertebrate species tested in the field assay, and of six additional invertebrates, we performed laboratory feeding assays under controlled conditions with a general predator. Carnivorous species suitable to evaluate palatability in laboratory assays are not abundant in Kongsfjord. The most conspicuous predatory organisms we observed feeding on epibenthic animals are the two starfish *Solaster endeca* (LINNAEUS, 1771) and *Crossaster*

papposus (LINNAEUS, 1767). Since both species could not be collected in sufficient numbers and kept successfully at laboratory conditions we used the starfish, *Asterias rubens* (LINNAEUS, 1758), from the North Sea as a model organism. This predator is distributed along the entire European Atlantic coast up to northern Norway, but does not reach Spitsbergen (Hayward & Ryland, 1990). *Asterias rubens* is a generalist feeder, known to prey mainly upon large epifaunal organisms, i.e. gastropods, bivalves, crustaceans, polychaetes, and also ascidians (Bosence, 1973; Gulliksen & Skaeveland, 1973; Anger et al., 1977). Specimens of *Asterias rubens* were collected around the island of Helgoland (North Sea) by SCUBA diving and maintained in laboratory aquaria (10°C seawater, 12/12 h light/dark cycle) at the Alfred Wegener Institute, Bremerhaven, on a maintenance diet of shrimp, fish and mussels. Starfish were starved for two days prior to feeding experiments. During these experiments animals were kept individually in plastic bowls (25 cm in diameter) filled with seawater. After two hours of acclimatization, one piece of invertebrate tissue to be tested for palatability (hereafter referred to as test food) was offered to each of 20 starfish by placing the animal on top of the bite-sized food item. These food items were obtained from several individuals of the same species. Care was taken to offer pieces of the same body part including surface tissue to each of the starfish. An equal number of starfish was offered a piece of control food (fish, *Sebastes* sp. CUVIER, 1829) at the same time. A food item was considered rejected as soon as a starfish moved away from the food. After each starfish had either rejected or consumed the respective food item but at least after eight hours, significant differences in consumption between test and control food were determined by Fisher's exact test (Sokal & Rohlf, 1981).

BIOCHEMICAL COMPOSITION

Palatability may, among others, depend on the nutritional quality of the prey tissue. Therefore, we analyzed the biochemical composition of the investigated species by measuring protein, lipid, carbon and nitrogen content. Frozen invertebrate tissue was freeze-dried and ground to a fine powder in an analytical mill. Samples were pooled from several individuals in order to obtain sufficient mass for the complete set of analyses. Hence, we provide average values per species for their

biochemical composition. Sub-samples of tissue powder were weighted and subjected to the following analysis based on well-established protocols.

(1) Soluble protein content was determined using a commercial Bradford (Bradford, 1976) protein test (Biorad) and bovine serum albumen as a standard. Samples of 10 mg DW were extracted for proteins by two different methods. i) For measuring NaOH-soluble protein samples were digested for 24 hours in 1ml 1N NaOH. One ml of 1N HCl was added to neutralize the solution of proteins. ii) Aqueous extracts were obtained by using 0.9% NaCl (w/v) and extracting tissue in an ultrasonic bath for one minute. Samples were measured in replicates of four (n=4) for both extraction methods.

(2) Lipid content was determined using a gravimetric technique described by Hagen (1988). Samples of 200 mg DW were extracted three times with a 2:1 mixture of dichloromethane-methanol in an ultrasonic bath. The supernatant was partitioned with 0.88% KCl-solution followed by centrifugation to separate lipids from the aqueous residue. The lipid phase was transferred into pre-weighed vials, evaporated to dryness under nitrogen and weighed (n=2).

(3) Carbon and nitrogen were measured according to the protocol of Verardo et al. (1990). Samples of 10-20 mg DW were packed airtight in tin foil and measured in an elemental analyser (elementar vario EL, Elementar Analysensysteme GmbH, Hanau) for carbon and nitrogen (n=4).

(4) Additionally, water content was determined by measuring subsequently fresh weight (FW) and dry weight (DW). Each of four replicates per species was blotted gently with tissue paper to remove external water and weighed thereafter. After freezing and lyophilizing samples were weighed again to determine water loss.

RESULTS

In situ assays for palatability were performed with ten invertebrate species, exposing tissue to natural consumer assemblages. From diving observations during the experiments we suggest that these assemblages mainly consisted of amphipods. The species tested were the sponges *Suberites ficus* LUNDBECK, 1902, *Haliclona rosea* (BOWERBANK, 1866) and *Spongosorites genitrix* (SCHMIDT),

the actinians *Hormathia nodosa* (FABRICIUS, 1780) and *Urticina eques* (GOSSE, 1860), the octocoral *Gersemia rubiformis* (EHRENBERG, 1834), the bryozoan *Alcyonidium gelatinosum* (LINNAEUS, 1761) and the ascidians *Halocynthia pyriformis* (RATHKE, 1806), *Styela* spp. and *Synoicum turgens* PHIPPS, 1774.

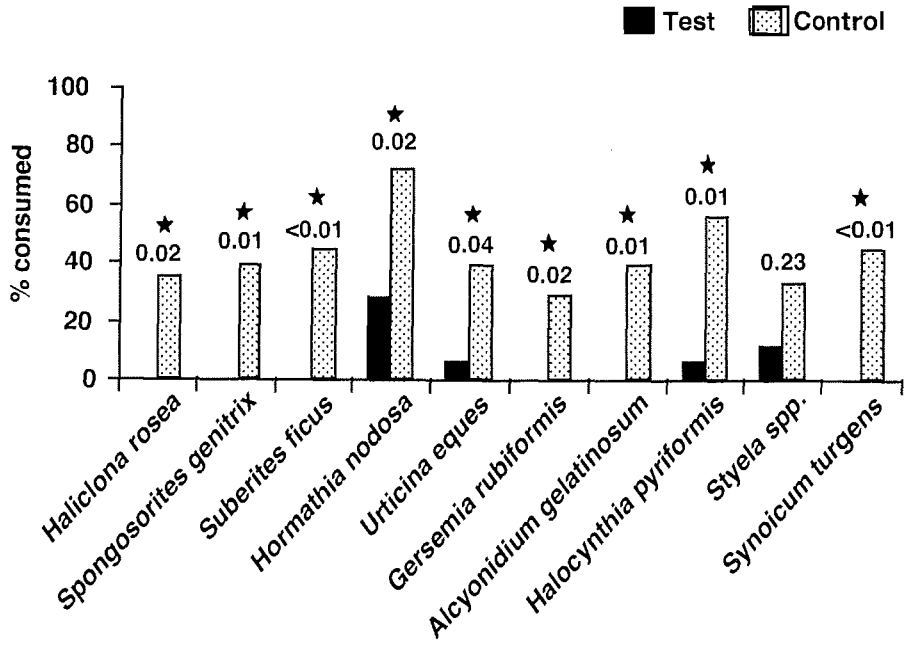

Figure 2: Field palatability assay with natural assemblages of predators: Percent pieces experimental and control tissue consumed. Statistical analysis was by Fisher's exact test. Asterisks indicate significant differences between test and control food. P values are given above bars.

With the exception of the ascidian *Styela* spp., all invertebrates were consumed significantly less compared to the control food offered simultaneously ($P<0.05$, Fig. 2). Six of the offered species were not preyed on at all while the control food was eaten to at least 29% in all experiments. Although *Styela* spp. was consumed less than the control as well, this difference was not statistically significant ($P=0.23$).

Results of palatability assays of sixteen invertebrate species against the predatory starfish *Asterias rubens* are shown in Fig. 3. These experiments were carried out under controlled laboratory conditions, and were very similar to the results obtained from the field assays.

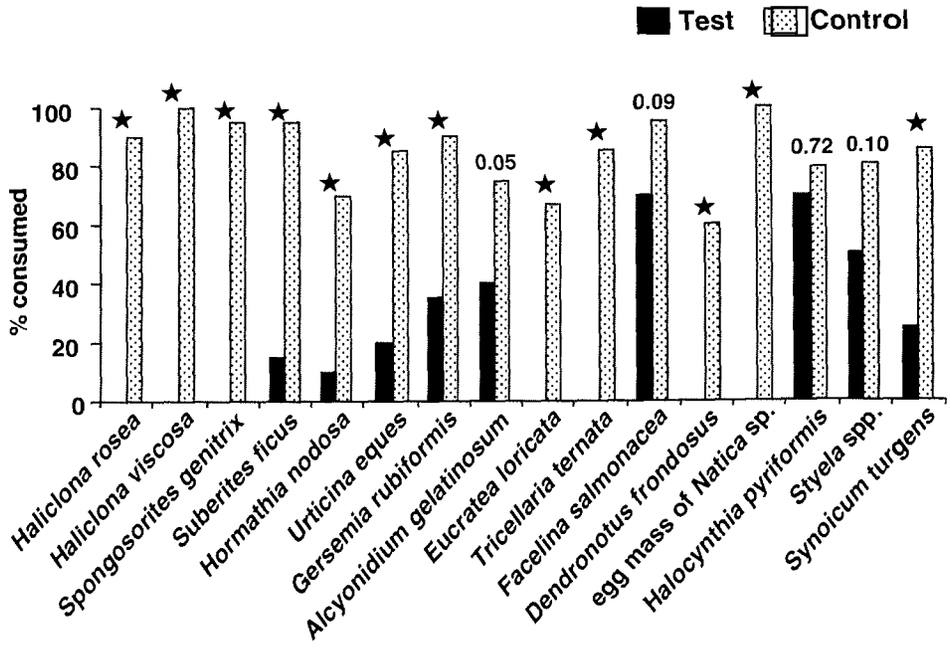

Figure 3 Starfish palatability assay under controlled laboratory conditions: Percent pieces experimental and control tissue consumed. Statistical analysis was by Fisher's exact test. Asterisks indicate significant differences ($P<0.001$) between test and control food, P values for not significant assays are given above bars.

From those species tested in field and laboratory assays, the ascidean *Halocynthia pyriformis* and the bryozoan *Alcyonidium gelatinosum* were rejected by the natural predator assemblage but did not significantly deter starfish feeding. In the laboratory assays, test food was always consumed less than the control food, but differences were not significant for the two ascidians *Halocynthia pyriformis* and *Styela* spp., the bryozoan *Alcyonidium gelatinosum* and the nudibranch *Flabellina salmonacea* (COUTHOUY, 1828) ($P>0.05$). The other twelve investigated invertebrate species significantly inhibited starfish feeding ($P\leq0.05$).

The biochemical composition of the investigated invertebrates and of the control food (*Sebastes* sp.), expressed as water content, lipid content, NaOH- and NaCl-soluble protein, nitrogen content, and as carbon to nitrogen ratio are given in Table 2.

Table 2: Biochemical parameters of invertebrates from Kongsfjord. Overall nutritional quality indicates subjective ranking based on all parameters measured.

Species	% FW	% DW				C/N-Ratio	Overall Nutritional quality
	Water (n=4)	Lipid (n=2)	NaCl soluble Protein (n=3-7)	NaOH soluble Protein (n=4)	Nitrogen (n=3)		
Haliclona rosea	89.0 ± 1.0	5.5	2.5 ± 0.2	5.7 ± 0.5	5.4 ± 0.4	4.7 ± 0.1	Medium
Haliclona viscosa	84.0 ± 1.8	6.9	0.7 ± 0.1	3.6 ± 0.3	5.2 ± 0.1	5.0 ± < 0.1	Medium - Low
Spongosorites genitrix	83.5 ± 1.6	4.3	1.7 ± 0.2	5.5 ± 0.2	4.4 ± 0.1	4.0 ± 0.1	Medium
Suberites ficus	82.1 ± 1.1	11.1	6.1 ± 0.8	7.6 ± 0.3	7.7 ± 0.2	4.9 ± < 0.1	High
Hormathia nodosa	84.9 ± 0.8	7.0	4.4 ± 0.4	6.0 ± 0.5	9.5 ± 0.1	3.8 ± < 0.1	High
Urticina aff. *eques*	91.1 ± 3.1	8.4	7.6 ± 0.6	6.9 ± 0.6	11.3 ± 0.1	3.5 ± < 0.1	High
Gersemia rubiformis	81.1 ± 2.3	5.1	1.5 ± 0.4	6.1 ± 0.5	6.8 ± 0.6	4.4 ± 0.2	Medium
Alcyonidium gelatinosum	90.9 ± 1.8	4.8	2.8 ± 0.2	5.0 ± 0.5	7.0 ± 0.1	3.7 ± < 0.1	Medium
Eucratea loricata	77.7 ± 0.3	2.1	1.1 ± 0.3	3.8 ± 0.2	3.6 ±<0.1	5.5 ± 0.1	Low
Tricellaria ternata	61.1 ± 3.6	0.8	0.7 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	8.9 ± 0.2	Low
Facelina salmonacea	92.7 ± 0.5	4.9	4.4 ± 0.3	ND	7.9 ± 0.4	4.1 ± 0.2	High
Dendronotus frondosus	93.1 ± 0.6	7.7	3.3 ± 0.1	ND	8.2 ± 0.2	3.8 ± < 0.1	High
Egg mass of *Natica* sp.	32.2 ± 2.3	0.1	0.1 ± < 0.1	-0.2 ± 0.1	0.4 ± 0.1	10.0 ± 1.5	Low
Halocynthia pyriformis	88.9 ± 2.1	5.3	1.3 ± 0.1	4.8 ± 0.6	5.6 ± 0.2	5.6 ± 0.1	Medium
Styela spp.	86.6 ± 0.8	4.3	2.7 ± 0.4	5.9 ± 0.3	6.4 ± 0.3	4.5 ± 0.2	Medium
Synoicum turgens	90.2 ± 0.5	4.1	0.9 ± 0.1	4.9 ± 1.0	3.9 ± 0.2	5.6 ± 0.1	Low
Sebastes sp. (control food)	ND	8.6	4.4 ± 0.8	8.3 ± 0.6	14.0 ± 0.1	3.6 ± 0.1	High

ND = not determined

For most of the investigated species the relative water content varied between 80% and 93% wet weight (WW), but was distinctively lower for the egg mass of *Natica* sp. SCOPOLI, 1777 (32.3%) and for the bryozoan *Tricellaria ternata* (ELLIS & SOLANDER, 1786) (61.9%). Lipid content ranged from a maximum of 11.1% dry weight (DW) in the sponge *Suberites ficus* to a minimum of 0.1% DW in the egg mass. In most other species lipid content averaged between 4-8% DW. NaCl-soluble protein levels were mostly between 1-4% DW, except for *Suberites ficus* (6.1% DW) and *Urticina* aff. *eques* (7.6% DW) which both had noticeably higher protein contents. NaOH-soluble protein ranged between zero (egg mass of *Natica* sp.) and 7.6% (*Suberites ficus*). Due to the extraction method values for NaOH soluble proteins were two to five times higher than NaCl-soluble proteins for most of the investigated species, but relative levels of protein among the species mostly remained the same. The only exception was *Gersemia rubiformis*; NaCl-soluble protein of this species was in a medium range compared to the other species, while NaOH-soluble protein was in a range of high protein content. Nitrogen was highest in *Urticina* aff. *eques* with 11.3% DW, and varied between 2-9% DW in the other species investigated. The values of biochemical parameters measured for

Sebastes sp. used as a control food ranged within the higher levels for all parameters measured, but were still within the range of some invertebrates like *Urticina* aff. *eques* and *Suberites ficus*. Considering all measured parameters, *Urticina* aff. *eques* and *Suberites ficus* showed highest values, followed by *Dendronotus frondosus* (ASCANIUS, 1774), *Flabellina salmonacea* and *Hormathia nodosa*. Apart from the egg mass of *Natica* sp., other species with relatively low biochemical parameters were *Tricellaria ternata*, *Eucratea loricata* (LINNAEUS, 1758), *Spongosorites genitrix* and *Synoicum turgens*. The other invertebrates showed medium values of lipid, protein and nitrogen, except the sponge *Haliclona viscosa* (TOPSENT, 1888), which contained a relatively high percentage of lipids (6.9% DW), a medium percentage of nitrogen (5.2% DW) but very low protein (0.7% DW).

DISCUSSION

NUTRITIONAL QUALITY

Within the trophic food web, most heterotrophic organisms can be regarded in two positions, the position of a consumer or that of a prey. For a consumer, biochemical composition of its body tissue, e.g. lipids, proteins, carbohydrates, can be an important measure of its dietary situation, i.e. if it is well fed or starved. For its position as a prey organism the biochemical body composition is an important measure of its nutritional quality as food for higher trophic levels. Different investigators used various parameters separately or in combination to evaluate this nutritional quality of invertebrates, such as insoluble or soluble protein, total lipid content, carbohydrates, ash content, chitin content, caloric content, or organic content (Carefoot, 1973; Larson et al., 1980; McClintock, 1987; Wacasey & Atkinson, 1987; McClintock et al., 1992; Chanas & Pawlik, 1995; Rogers et al., 1995; Slattery & McClintock, 1995; Stachowicz & Lindquist, 2000; Granado & Caballero, 2001). The variety of parameters measured and different methods employed make comparisons difficult. Most literature data comparable to our measurements refer to Antarctic invertebrates. McClintock (1987) measured NaOH-soluble protein and lipid content of 17 sponges from McMurdo Sound,

Antarctica, and he reports values between 5.0-19.7% DW and 2.1-9.1% DW, respectively. NaOH-soluble protein content of sponges from Kongsfjord (3.6-7.6% DW) is hence within the lower range whereas lipid content is in a similar range (4.3-11.7% DW) as in Antarctic sponges. Particularly higher in NaOH-soluble protein as well as lipid content is the Antarctic soft coral, *Gersemia antarctica*, (20.7% DW and 12.6% DW, respectively; Slattery & McClintock, 1995) compared to the closely related *Gersemia rubiformis* from Kongsfjord (6.1% DW and 5.1% DW, respectively). Protein and lipid content for the other invertebrates measured (see Table 2) are in a similar range as those for sponges and corals from Kongsfjord discussed above, thus in a lower range compared to species from Antarctica. Since total caloric content is mostly associated with protein content (McClintock, 1987; McClintock et al., 1992), and protein content in our samples was comparatively low, invertebrates from Kongsfjord seem to exhibit overall relatively low total energy contents. Caloric contents reported for invertebrates from the Canadian Arctic (Wacasey & Atkinson, 1987) are in the lower range compared to those from the Antarctic, and hence are probably more in agreement with ours.

Beside interspecific variability, intraspecific variation in nutritional quality, e.g., due to age, may also be expected but has not been investigated in this study. The pooled samples of whole body tissue used here may smooth individual variation and hence estimate nutritional quality of the species rather than the individuals. However, nutritional variation among body parts, e.g. between surface and sub-surface tissue, might be an important issue for a predator in prey selection (Penney, 2002) and should be further investigated in future studies.

FEEDING BIOASSAYS

Regular observations by divers during our *in situ* experiments suggest that mostly amphipods were feeding on the offered food. While gastropods (*Buccinum* sp.), moving towards the experimental set up, could not reach the tissue cubes hanging at least 5 cm above the ground, amphipods were attracted and started feeding immediately. It is unlikely that fish species were feeding on the experimental food since there have been only very few observations of fishes during over 200 SCUBA dives in the Kongsfjord. However, we cannot exclude fish completely as

consumers in the *in situ* experiments, because some fish species are reported from the shallow water (< 30 m) of Kongsfjord with mainly pleuronectids feeding on epibenthic fauna (Hop et al., 2002) The most abundant amphipod species we captured in Kongsfjord using traps baited with fish were the lysianassids *Anonyx nugax* (PHIPPS, 1774) and *Onisimus edwardsi* (KRØYER, 1846), indicating that these species were also the main groups feeding on the experiments. Many lysianassid taxa are known for their scavenging feeding behaviour but have also been reported to attack living animals in traps (references in Slattery & Oliver, 1986), as well as injured and dislodged benthic fauna (Slattery & Oliver, 1986) and zooplankton (Legezynska, 2001). Their feeding habits are often opportunistic and seem to depend on many factors like season or age, e.g. small individuals of *Anonyx nugax* prey on different pelagic and benthic animals and also use detritus (Weslawski et al., 1991; Dr. Legezynska, Institute of Oceanology, Sopot, Poland, personal communication). It is likely that necrophagy is an adaptation to food-limited environments (McClintock, 1994) and has been viewed as a component of a more general feeding strategy in e.g. Antarctic echinoderms (Warner, 1982). Therefore we assume lysianassid amphipods to be suitable indicator organisms for palatability of invertebrate species in our field assays. If invertebrate tissues offered in the field are not protected by any defensive mechanism (see below) they should be a suitable food source for an opportunistic feeder.

To compare our *in situ* experiments with experiments under controlled conditions we performed feeding experiments in the laboratory. *Anonyx nugax*, the major predator during the *in situ* experiments, was not a suitable organism for our laboratory assays because their feeding efficiency on the invertebrate tissues offered as food is likely to vary with size, shape and texture of the tissue and will probably confound quanitative measures. According to Hop et al. (2002) starfish are the most conspicuous carnivorous invertebrates in Kongsfjord beside prosobranch snails. While carnivorous starfish like *Solaster endeca* and *Crossaster papposus* are common in the outer Kongsfjord, we mostly found *Henricia sanguinolenta* (MÜLLER, 1776) and *Poraniomorpha hispida* (SARS, 1872) in the inner part, which mainly feed on suspended materials and detritus, respectively (references in Jangoux, 1982; personal observation). Because *Solaster endeca* und *Crossaster papposus* could not be caught and kept in sufficient numbers under laboratory conditions, the starfish *Asterias rubens* from

the North Sea was chosen as a model bioassay organism. The taxonomically close *Asterias amurensis* can be a major predator on benthic communities in other Arctic locations (Fukuyama & Oliver, 1985). Despite the fact that *A. rubens* is not a sympatric predator on invertebrates in Kongsfjord, and that it has a different feeding method than amphipods by extruding their stomach over a whole piece of food rather than biting small pieces, results of feeding preferences were very similar. These similar preference / avoidance reactions of both predators towards the invertebrate species tested indicate that causes for prey selection in this case are of a more general nature and are beyond local adaptations.

The *in situ* feeding assays allow us to examine and quantify the feeding response of a natural assemblage of predators and scavengers towards tissues of benthic invertebrates. They also may give limited insight into predation intensity in our study area. In each of our *in situ* feeding assays a large quantity (29-72%) of the control food was consumed, however, in none of the experiments the controls were eaten entirely. This could indicate that natural assemblages of consumers had been saturated after a limited feeding period, probably because their abundance is generally relatively low in the middle and inner Kongsfjord. We found, however, significantly less consumption on most of the invertebrate tissues (0-28%, $P \leq 0.04$ for *Urticina* aff. *eques*, $P \leq 0.02$ for others). These results clearly show that some predators are present in the Kongsfjord, and that they are able to discriminate significantly between the different food sources. Nine out of ten invertebrate species were rejected significantly by naturally occurring predators, indicating that 90% of the species possess a defensive mechanism against predation. Also, 75% invertebrate species tested in the laboratory assay deterred feeding by the starfish *Asterias rubens*. Both data sets indicate that species from Arctic regions are unpalatable and defended against predators, probably on a comparably high level as species from temperate, tropical or Antarctic regions (McClintock, 1987; Paul, 1992).

The defensive options available to marine organisms are diverse, including nutritional quality (McClintock, 1987; Duffy & Paul, 1992; Chanas & Pawlik, 1995; Granado & Caballero, 2001), structural defenses (Steneck & Watling, 1982; Littler et al., 1983; Harvell, 1984; Van Alstyne et al., 1992; Chanas & Pawlik, 1995; Koh et al., 2000; Puglisi et al., 2000), tissue toughness (Chanas & Pawlik, 1995), temporal or spatial avoidance of predators (Rogers et al., 1995), or chemical

defenses (Faulkner & Ghiselin, 1983; Sammarco & Coll, 1992; Van Alstyne & Paul, 1992; Schupp et al., 1999; Assmann et al., 2000; Puglisi et al., 2000; Amsler et al., 2001). Following we will discuss in detail the nutritional quality of the investigated invertebrate species as a potential defense mechanism, while chemical and other defenses will be discussed elsewhere (Lippert, in prep.).

EFFECT OF NUTRITIONAL QUALITY ON PREDATION

According to the optimal foraging theory (Hughes, 1980; Pyke, 1984) it would be predicted that species with higher nutritional value should be preferred prey compared to prey with lower nutritional values. Although invertebrates from the Kongsfjord seem to have relatively low nutritional values compared to invertebrates from other geographic regions (see above), different levels of nutritional quality can be distinguished among the taxa measured in this study. The investigated species can be grouped into three categories, according to their overall nutritional quality, based on the biochemical parameters proteins, nitrogen and lipids (see Table 2). If nutritional quality of prey is a key factor in food selection of predators, we would expect to see differences between feeding rates of natural assemblages as well as of the starfish *Asterias rubens* on high, medium and low nutritional quality invertebrates.

Duffy & Paul (1992) present evidence from field assays that variation in nutritional quality among tropical reef species is of equally high importance in affecting an organism's susceptibility to predators as chemical defense. One practical implication from their results is that control food used in tests for feeding deterrence should match the test organism's nutritional composition as closely as possible. The fish used as control in our experiments ranged within the higher level for all measured nutritional parameters compared to the invertebrate species tested. In the *in situ* feeding assays predators were offered both test and control food simultaneously. Those cases where invertebrate test tissue was rejected might be due to a lower nutritional quality compared to the control food. From this experimental design we cannot conclude whether the natural assemblage of predators would have consumed invertebrate tissue of lower nutritional value would it have been offered alone. As mentioned above, the control food was never completely eaten after 24 hours, indicating that there was a surplus of food for the

number of predators present. However, some feeding preferences observed in our *in situ* experiments cannot be explained by the higher nutritional value of the control food. The sponge *Suberites ficus* had a nutritional value comparable to the control food and was still not consumed. Therefore, the unpalatability of *Suberites ficus* must have reasons other than nutritional quality. Sponges are often rich in secondary metabolites involved in chemical defense (McClintock & Gauthier, 1992; Baker et al., 1993; McClintock et al., 1993; Harper et al., 2001), or have silicious or calcareous spicules that may deter feeding. Similarly, both actinian species, *Urticina* aff. *eques* and *Hormathia nodosa*, had relatively high nutritional values and were eaten significantly less than the control food. If not through low nutritional quality, cnidarians can also be defended through protective nematocysts or by chemical defenses (Stachowicz & Lindquist, 2000). In contrast, the ascidian *Styela* spp. was readily consumed although its nutritional value was ranging only in a medium to lower level compared to the control food. Consequently, there is no consistent correlation between the rejection or consumption of invertebrate tissue in the *in situ* feeding assay and the nutritional quality. We cannot exclude that low nutritional quality may be the reason for less consumption in those cases where invertebrate tissue exhibited lower nutritional value than control tissue, e.g., the colonial ascidian *Synoicum turgens*. Nutritional quality, however, is not likely to be the reason for unpalatability in those invertebrate tissues of high nutritional value, e.g., the sponge *Suberites ficus* and the actinians *Urticina* aff. *eques* and *Hormathia nodosa*.

In contrast, *Asterias rubens* was offered only one food item at any given time and could not choose between various food items of different nutritional quality. In this experimental design the high quality of the control food should not conceal any feeding preference or avoidance towards test tissues. Differences in palatability of invertebrate tissues between both assays employed were detected for the bryozoan *Alcyonidium gelatinosum* and the ascidian *Halocynthia pyriformis*. While these two species were not consumed during the *in situ* experiments, no significant differences in consumption between test and control tissue were found within the starfish assay. Possibly, nutritional quality of the bryozoan and the ascidian was low compared to the fish control in the experiment where there was a choice but still sufficient (medium range) for the starfish to feed when there was no choice. On the other hand, four other species were in the same range of nutritional

quality as *Alcyonidium gelatinosum* and *Halocynthia pyriformis* and were either rejected (*Gersemia rubiformis*, *Haliclona rosea*, *Spongosorites genetrix*) or readily eaten (*Styela* spp.) in both assays.

Several other invertebrate species with low nutritional values (e.g., the bryozoans *Eucratea loricata* and *Tricellaria ternata*, the egg mass of *Natica* sp. and the ascidian *Synoicum turgens*) were not consumed by the starfish. This might be an indication of reduced palatability due to low nutritional quality. On the other hand, these bryozoans are heavily calcified which may act as a structural defense (Harvell, 1984). In contrast, *Alcyonidium gelatinosum*, which was not significantly rejected by the starfish, is less calcified and has a 2-4 fold higher nutritional value than *Tricellaria ternata* and *Eucratea loricata*. Therefore, the higher palatability of *Alcyonidium gelatinosum* could either be due to higher nutritional quality or to less structural defense. The tunic of the ascidian *Synoicum turgens* mainly consists of cellulose-like polysaccharides, which are largely undigestible to animal predators. Similar to the *in situ* experiments, some invertebrate species with relatively high nutritional quality were not consumed by *Asterias rubens*, e.g. *Suberites ficus*, *Hormathia nodosa*, *Urticina* aff. *eques* and *Dendronotus frondosus*. The only species with a high nutritional value consumed by the starfish was *Flabellina salmonacea*.

We conclude that for most of the species tested the predictions of the optimal foraging theory are not met because there is no obvious positive correlation between nutritional quality and feeding in laboratory and *in situ* experiments. We cannot exclude that low or high nutritional quality of some invertebrates may be the reason for rejection or consumption, respectively, by the predators employed in our assays. It is likely, however, that in most cases other mechanisms than nutritional quality are affecting palatability of the invertebrate species tested. This is in accordance to the findings of McClintock (1987) and McClintock et al. (1992) for Antarctic sponges and nudibranchs, respectively. These authors could not observe a correlation between nutritional quality of prey and predation rates. They also conclude that other defense mechanisms or biochemical parameters not measured (e.g. vitamins or minerals) may be responsible for the observed feeding patterns. Similar results were obtained for carnivorous and herbivorous feeding from different geographic regions measuring various parameters for nutritional value (Chanas & Pawlik, 1995; Granado & Caballero, 2001; Stachowicz &

Lindquist, 2000; Larson et al., 1980; Carefoot, 1973; Rogers et al., 1995). Many organisms also combine several types of defenses (Harvell et al., 1988; Van Alstyne & Paul, 1992; Van Alstyne et al., 1994; Stachowicz & Lindquist, 2000) and nutritional quality, although important, may be masked by other defense mechanisms such as structural or chemical defenses.

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PUBLICATION II

CHEMICAL DEFENSE AGAINST PREDATORS IN A SUB-ARCTIC FJORD

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ABSTRACT

The development of chemical defences in marine organisms is supposed to be driven by intense pressure of predation and competition. While benthic communities in tropical and also Antarctic regions are thought to be mainly structured by intra- and interspecific interactions, these factors are proposed to be less important in northern high latitudes. Consequently, selective pressure for chemical defence should be low in these regions. To investigate the incidence of chemical defence in northern high latitudes, crude extracts of 18 abundant sessile or slow moving invertebrate species (4 sponges, 3 actinians, 1 soft coral, 4 bryozoans, 3 ascidians and the egg mass of a gastropod) from Kongsfjord (Spitsbergen) were tested for feeding deterrent activity. Laboratory assays were performed by offering artificial food with extracts to two different predators, the amphipod *Anonyx nugax* which is a common species in Kongsfjord, and the starfish *Asterias rubens* from the North Sea. Two of the 18 extracts tested (*Haliclona viscosa*, *Hormathia nodosa*), exhibited significant feeding deterrent effects in the amphipod assay. Furthermore, six extracts had a significantly stimulating effect on the amphipod feeding, and ten extracts did not affect consumption. In the starfish assay only the crude extract of *Haliclona viscosa* was significantly rejected. For *Haliclona viscosa*, feeding deterrence could be established for two pure compounds, and for *Hormathia nodosa* for one fraction. The data indicate that feeding deterrent compounds are present in sub-Arctic marine invertebrates but are less abundant than in temperate, tropical and Antarctic species. At this point it seems that predation is not a strong enough selective pressure to drive the development of chemical defences in a large variety of invertebrates.

Keywords Arctic; *Anonyx nugax*; *Asterias rubens*; chemical ecology; feeding deterrence; invertebrates

INTRODUCTION

Benthic communities in northern high latitudes are commonly considered to be less diverse than those from lower latitudes, especially from tropical regions, but also from the Antarctic (Kendall, 1996; Gray, 2001). Not all investigations, however, confirm this pattern and, locally, Arctic communities can be rich in biomass and diversity (Kendall, 1996; Brandt, 1997). At lower latitudes, high biodiversity and the resulting influence of biological interactions such as predation, grazing and competition in benthic communities have been suggested to drive the development of chemical defences in many sessile or slow moving invertebrates and macroalgae. Mainly the intense predation and herbivory by fishes in tropical regions is thought to have resulted in high selection for noxious and toxic chemical compounds in tropical marine organisms (Bakus and Green, 1974; Green, 1977; Hay and Fenical, 1988; Hay and Steinberg, 1992). Early investigations on the toxicity of marine invertebrates led to a latitudinal hypothesis, suggesting an inverse correlation between the incidence of chemical defence and latitude (Bakus and Green, 1974). More recently, the simplistic version of this hypothesis has come into question, since it has been found that also within-region variance can be particularly high (Bolser and Hay, 1996). Moreover, for the southern hemisphere several authors showed that the suggested latitudinal relationship is invalid (McClintock, 1987; Avila et al. 2000; Amsler et al., 2001). The incidence of toxicity in Antarctic sponges is similar to that in tropical and much higher than in temperate species (McClintock, 1987). Already Dayton et al. (1974) suggested that Antarctic benthic communities are "biologically accommodated" and are structured mainly by biological factors such as predation and competition, favoured by the stable physical conditions below the zone of ice scour. In contrast, Arctic and sub-Arctic benthic communities are supposed to be mainly structured by physical factors, such as wave action, ice gouging (Gutt et al., 1996), and the influence of glaciers and river runoffs, adding high loads of inorganic material and freshwater mainly to inner-fjord locations (Wlodarska-Kowalczuk et al., 1998). Additionally, it is suggested that the relatively young evolutionary history of the Arctic Ocean contributes to a low biodiversity due to a relatively short period for adaptation and speciation (Gray, 2001). Although, it has been shown that locally predation by walruses (Oliver et al., 1985) or grey whales (Highsmith & Coyle, 1992) can have

a significant impact, predator prey relationships among lower trophic levels, are relatively unstudied in Arctic waters, especially within the less abundant hard bottom communities. Large demersal fishes are not common in northern waters (Dayton et al., 1994) and investigations by Thorson (1957) and Gulliksen (1979) found predation on Arctic benthic communities in general to be sparse. Therefore selective pressure for chemical defences against predators may be assumed to be low.

This is one of the first attempts to address chemical defence against predation at high northern latitudes. Considering the above mentioned evolutionary history, as well as physical and biological factors of the Arctic environment, we hypothesise that chemical defences are poorly developed in benthic invertebrates of northern high latitudes. To test this hypothesis we screened palatability of organic extracts of abundant sessile or sluggish invertebrate species from Kongsfjord, Spitsbergen, to two different types of predators. Screening across a wide range of systematic groups (sponges, actinians, octocorals, nudibranchs, ascidians, bryozoans and the egg mass of a gastropod) is expected to give a good indication of the distribution and abundance of chemical defences in the study area.

MATERIAL AND METHODS

STUDY SITE AND SAMPLING

Seventeen abundant sessile or slow moving invertebrate species and one egg mass of a gastropod were collected by SCUBA diving in Kongsfjord, a glacial fjord at the northwestern part of Spitsbergen (79°N, 12°E) (Fig. 1), in the summers of 1999, 2000 and 2001. The four sampling sites within the fjord (Kongsfjordneset, Hansneset, a cave close to Hansneset, Prinz Heinrich Islands) are shown in Fig. 1. A detailed description of the fjord is given in Lippert et al. (2001). Investigated species and the according sampling sites are listed in Table 1. Later identification showed that the collection of the ascidian *Styela* spp. consists of a mixture of the two morphologically extremely similar species *Styela rustica* (Linnaeus) and *Styela gelatinosa* Traustedt. Samples were immediately brought to the laboratory and

fouling organisms, when present, were removed from the surface of the invertebrates. Specimens were shock frozen in liquid nitrogen, lyophilised and stored at –28°C for later extraction. Voucher specimens were preserved in 5% formaldehyde-seawater solution for later species identification.

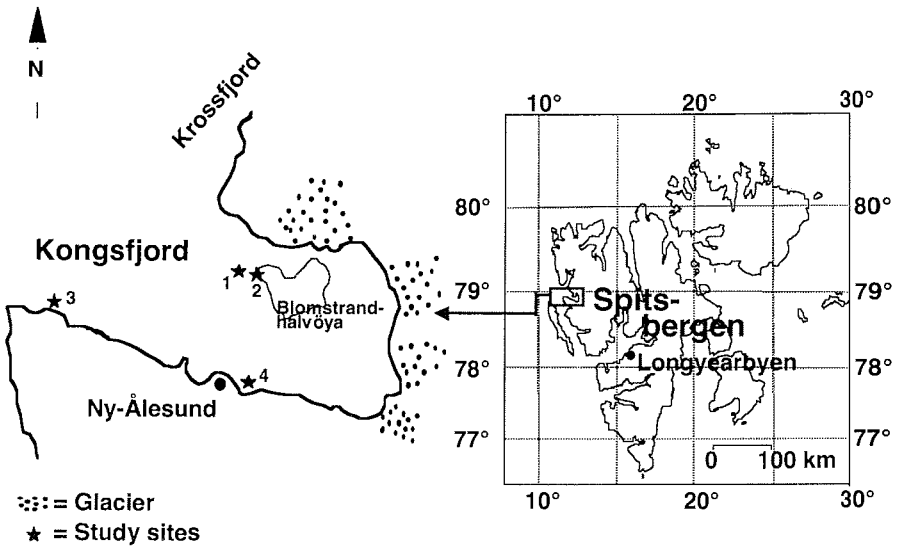

Figure 1: General view of Spitsbergen, showing Kongsfjord and the study sites. Asterisks and numbers indicate the different sampling sites (1-4): 1 – Hansneset; 2 – Cave; 3 – Kongsfjordneset, 4 – Prinz Heinrich Islands.

CRUDE EXTRACTS, FRACTIONATION AND ISOLATION

To obtain crude extracts a known mass of freeze-dried tissue from each species was grounded by mortar and pestle and extracted repeatedly in 1:1 methanol:dichloromethane, or subsequently in methanol, 1:1 methanol:dichloromethane, and dichloromethane. The partial extracts were combined (see Table 1 for details). We used pooled samples from several individuals in order to obtain sufficient mass. The organic extracts were filtered to remove particles, and extracts were concentrated under reduced pressure by rotary evaporation at low heat (40°C). Crude extracts were transferred into pre-weighted vials, evaporated to dryness under nitrogen or vacuum, and weighted. The extract yield per g DW (Table 1) is referred to as the natural concentration and

was used to calculate the amount of extract used in feeding assays (see below). All crude extracts were stored at –28°C until use in feeding experiments.

Extracts of those species that showed activity in feeding assays were fractionated further, i.e. the actinian *Hormathia nodosa* (Fabricius) and the sponge *Haliclona viscosa* (Topsent). The crude extract of *Hormathia nodosa* was partitioned subsequently with 2,2,4-trimethylpentane, *n*-butanol (BuOH), and water. The crude extract of *Haliclona viscosa* was partitioned between methanol and *n*-hexane. The methanol soluble part was dried and brought up in water, and was then partitioned subsequently with ethyl acetate (EtOAc) and *n*-BuOH. These latter fractions (EtOAc and *n*-BuOH) were combined after HPLC analysis revealed a similar chemical composition. The remaining water fraction was also subjected to bioassays. The active *n*-hexane and EtOAc/*n*-BuOH fractions of *Haliclona viscosa* were further purified with low-pressure liquid chromatography (silica gel, hexane:EtOAc 6.5:3.5) and preparative RP-HPLC using a Kromasil RP 18 column (4.6 x 250 mm, 5 μm particle size), respectively.

Table 1: Investigated invertebrate species, sampling site and depth of sampling, extraction method and natural concentration. A indicates extraction in 1:1 MeOH/DCM, B indicates subsequent extraction in 100% MeOH, 1:1 MeOH/DCM, 100% DCM. Natural concentrations are given in g extract/gDW.

Species	Sampling site	Depth of sampling [m]	Extraction method	Natural concentration [g/gDW]	Amphipod assay	Starfish assay
Haliclona viscosa	Hansneset	15-25	B	0.23	X	X
Haliclona rosea	Hansneset	10-25	B	0.18	X	X
Suberites ficus	Cave	4-5	A	0.24	X	X
Spongosorites genitrix	Cave	4-5	A	0.23	X	X
Hormathia nodosa	Hansneset	10-25	A	0.23	X	X
Urticina eques	Kongsfjordneset	10-25	A	0.27	X	X
Urticina asiatica	Kongsfjordneset	10-25	A	0.19	X	X
Gersemia rubiformis	Cave	2-4	A	0.23	X	X
Tricellaria ternata	Hansneset	1-10	A	0.08	X	X
Eucratea loricata	Hansneset	1-6	B	0.12	X	X
Crisiella sp.	Different sites	1-6	A	0.10	X	X
Alcyonidium gelatinosum	Hansneset	1-8	A	0.33	X	X
Natica sp. (egg mass)	Different sites	4-30	A	0.02	X	X
Dendronotus frondosus	Different sites	1-25	A	0.50	X	-
Flabellina salmonacea	Different sites	1-25	A	0.45	X	-
Halocynthia pyriformis.	Hansneset	5-25	B	0.24	X	X
Styela spp.	Prinz Heinrich Islands	15-30	B	0.28	X	X
Synoicum turgens	Hansneset	1-7	A	0.13	X	X
Sebastes sp.			A	0.17	X	X

ARTIFICIAL FOOD

To test their palatability, crude extracts, fractions or pure compounds were incorporated into artificial food, and offered to general predators in laboratory assays (see below). As a feeding stimulant we used freeze-dried and finely powdered fish (*Sebastes* sp. Cuvier). Crude extract, fractions or compounds were added in a small volume of solvent at natural concentrations (Table 1) to the powdered fish and then the solvent was removed from the mixture by rotary evaporation until dryness. For control food the fish was treated with an equivalent amount of solvent only. The artificial food pellets were prepared by heating 60 ml of seawater and mixing in 1.8 g alginic acid (Sigma). The mixture was allowed to cool to room temperature (RT), and the feeding stimulant (control) or the feeding stimulant coated with an extract to be tested (test) was added. For starfish assay pellets we added 749 mg powdered fish to 5.6 ml of liquid RT alginic acid; 560 mg powdered fish were added for amphipod assay pellets. The mixture was spread out in a petridish (54 mm in diameter), carefully covered with 15 ml of cold 1 M $CaCl_2$ solution and allowed to harden over night. Pellets of 6 mm in diameter were cut using a cork bore. Pellets containing organic crude extracts, fractions or compounds are hereafter referred to as test pellets. Pellets without any extract are referred to as control pellets.

AMPHIPOD FEEDING ASSAYS

Amphipod feeding experiments were performed with the abundant species *Anonyx nugax* (Phipps) from Kongsfjord. According to Weslawski et al. (1991), *Anonyx nugax* is a common arctic-boreal species, which is necrophagous and most likely also carnivorous. Its feeding habit seems to be opportunistic and to depend on many factors like season or age, e.g. small individuals of *Anonyx nugax* prey on different pelagic and benthic animals and also use detritus (Weslawski et al., 1991; Dr. Legezynska, Institute of Oceanology, Sopot, Poland, personal communication). In previous field assays we have observed *Anonyx nugax* to be a major predator in this area (Lippert and Iken, in prep.). Numerous individuals of this species were caught by traps baited with fish and kept in laboratory aquaria (4-6°C seawater) at Ny Ålesund research station, on a maintenance diet of fish. For feeding

experiments, in each of eight containers (1 l plastic beakers) 20 amphipods were placed. Each container held one test pellet and one control pellet. To determine changes in pellet mass caused by water uptake or loss of material during the experiment due to container effects, an equal number of beakers without amphipods contained also one test and one control pellet (control containers). In order to distinguish between control and test pellets food colouring was added to one of the pellets. All pellets were gently blotted and weighed before the experiments. Amphipods were removed when about half of the pellets in the experimental containers had been consumed, but latest after 8 hours. Since a choice experiment is no longer a choice experiment as soon as one pellet has been completely consumed, replicates in which only one pellet was left at the end of the experiment had to be excluded from statistical analysis. After the experiment, all test and control pellets were blotted again and weighed to determine mass changes during the experiments. Crude extracts of eighteen species, extract fractions of two species and pure compounds of one species were tested for palatability in this assay. To exclude effects of added food colouring on feeding preferences, preliminary experiments were performed following the experimental setup described above. Two pellets, one with and one without colour, but both without extract, were added to each experimental and each control container. No significant differences in consumption between coloured and uncoloured pellets were found ($P=0.613$).

Data were analysed according to Peterson and Renaud (1989). For each pellet, the change in mass was determined by subtracting the final mass from the initial mass. Then the change in mass of the test pellet was subtracted from the change in mass of the control pellet in the same container to give a single value for each container. The resulting values for test and control containers were transformed by double square route and tested for normal distribution using a Lillefors test ($\alpha=0.05$). If the requirement of normality was met we used a Student's t-test ($\alpha=0.05$) to test for differences between pellet mass change in test (with amphipods) and control (without amphipods) experiments. If data were not normally distributed we applied a non-parametric Wilcoxon test (Sokal and Rohlf, 1981).

Starfish feeding assays

In a second assay we tested palatability of crude extracts, fractions or compounds of 16 invertebrate species from the Kongsfjord against the starfish *Asterias rubens* (Linnaeus) (Table 1). Starfish exhibit a different feeding mode than amphipods by extruding their cardiac stomach over a food item and digest externally rather than biting small pieces from a food item like amphipods do. Since there are no carnivorous starfish in the inner Kongsfjord which could be caught and kept successfully at laboratory conditions in sufficient numbers, we chose the widely distributed starfish *Asterias rubens* from the North Sea. *Asterias rubens* was collected around the island of Helgoland (North Sea) by SCUBA diving and maintained in laboratory aquaria (10°C seawater, 12/12 h light/dark cycle) at the Alfred Wegener Institute, Bremerhaven, on a maintenance diet of shrimp, fish and mussels. Starfish were starved for two days prior to feeding experiments. During the experiments animals were kept individually in plastic bowls (25 cm in diameter) filled with seawater. After two hours of acclimatisation we first offered the starfish a control pellet by placing the animal on top of it. If the control pellet was consumed, the same starfish was then offered a test pellet. If the test pellet was rejected, we offered the starfish a second control pellet to determine whether the test pellet was rejected because the starfish was satiated. If a starfish consumed a control pellet after rejecting a test pellet, we concluded that the test pellet was rejected due to the added crude extract, fraction or compound.

A pellet was considered rejected as soon as a starfish crawled away from the food. Significant differences in consumption between test and control pellets were determined by Fisher's exact test (α=0.05, Sokal and Rohlf 1981). Only those replicates were included in the analysis in which both the first and the second control pellet were eaten.

RESULTS

Results of palatability assays with *Anonyx nugax* of crude extracts of seventeen invertebrate species and the gastropod egg mass are given in Fig. 2. A significant effect of crude extracts at natural concentrations on amphipod feeding was

observed for eight of these species. The extracts of the sponge *Haliclona viscosa* and the actinian *Hormathia nodosa* significantly deterred amphipod feeding ($P \leq 0.0001$), whereas the addition of crude extracts from the sponge *Suberites ficus* Lundbeck the actinians *Urticina eques* (Gosse) and *U. asiatica* (Averincev), the bryozoan *Alcyonidium gelatinosum* (Linnaeus), the ascidian *Styela* spp., and the *Natica* sp. Scopoli egg mass had a significantly stimulating effect on amphipod feeding ($P \leq 0.02$). Crude extracts of the remaining ten species had no significant effect on the consumption by amphipods at natural concentrations compared to consumption on the control pellets.

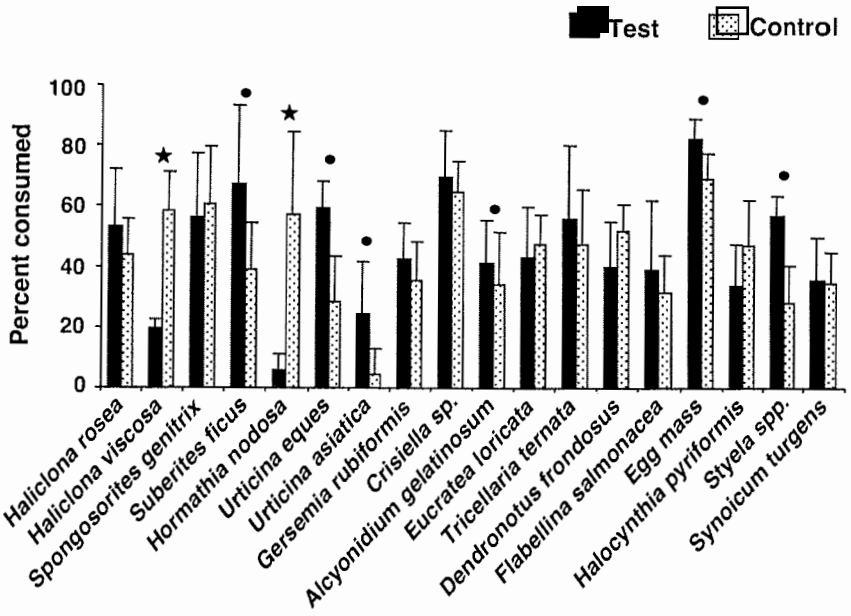

Figure 2: Percentage (mean ± SD) of test and control pellets consumed in palatability assays with the amphipod *Anonyx nugax*. Significant differences ($\alpha < 0.05$, Student's t-test) between consumption on test and control pellets are indicated by asterisks and circles: Asterisks indicate inhibition, circles promotion of feeding by crude extracts. N was between 7 and 8 for all experiments.

Extracts of the sponge *Haliclona viscosa* and the actinian *Hormathia nodosa* both of which were significantly rejected by the Amphipods were fractionated further. The crude extract of *Haliclona viscosa* was separated into three fractions, yielding an *n*-hexane, EtOAc/*n*-BuOH and aqueous remain fraction. 2,2,4-

trimethylpentane, *n*-BuOH and water were used for separation of the extract of *Hormathia nodosa*. The *n*-BuOH or EtOAc/*n*-BuOH soluble compounds from both species were significantly deterrent to *Anonyx nugax* (P≤0.001 *Haliclona viscosa*, and P=0.003 *Hormathia nodosa*), whereas both water-soluble fractions caused no effects (Fig. 3 and 4). For *Haliclona viscosa* also the *n*-hexane soluble compounds had an inhibiting effect on amphipod feeding (P=0.021), but no effect was observed from the 2,2,4-trimethylpentane fraction of *Hormathia nodosa* (Fig. 3 and 4). Further purification of deterrent fractions of the sponge *Haliclona viscosa* resulted in three pure compounds from the *n*-BuOH soluble material (X2, X3 and X4) and in two pure compounds (K1 and K2) as well as two mixed fractions containing several compounds (K1,2 and K3) from the *n*-hexane fraction. Results of amphipod feeding experiments with *n*-BuOH and *n*-hexane soluble compounds of *Haliclona viscosa* are shown in Fig. 4. Of the *n*-hexane soluble fraction, K2 and K1,2 showed feeding deterrent activity (P≤0.001 and P=0.034, respectively). Since K 1,2 is a mixture of compounds K1 and K2, and K1 alone does not show any deterrent activity, feeding inhibition is likely to be caused by compound K2. Of the *n*-BuOH soluble compounds only X2 caused significant effects on amphipod feeding (P=0.002).

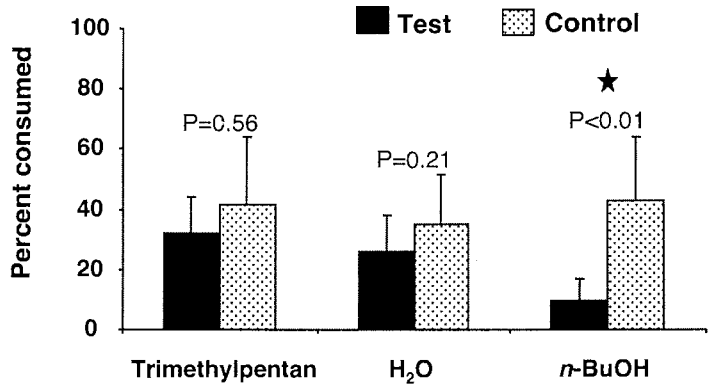

Figure 3: Consumption (mean percentage ± SD) of pellets treated with fractions and compounds of the actinian *Hormathia nodosa* in palatability assay with amphipods. Asterisks indicate significant differences (α<0.05, Student's t-test) between consumption of test and control pellets. P-values above bars, N=8.

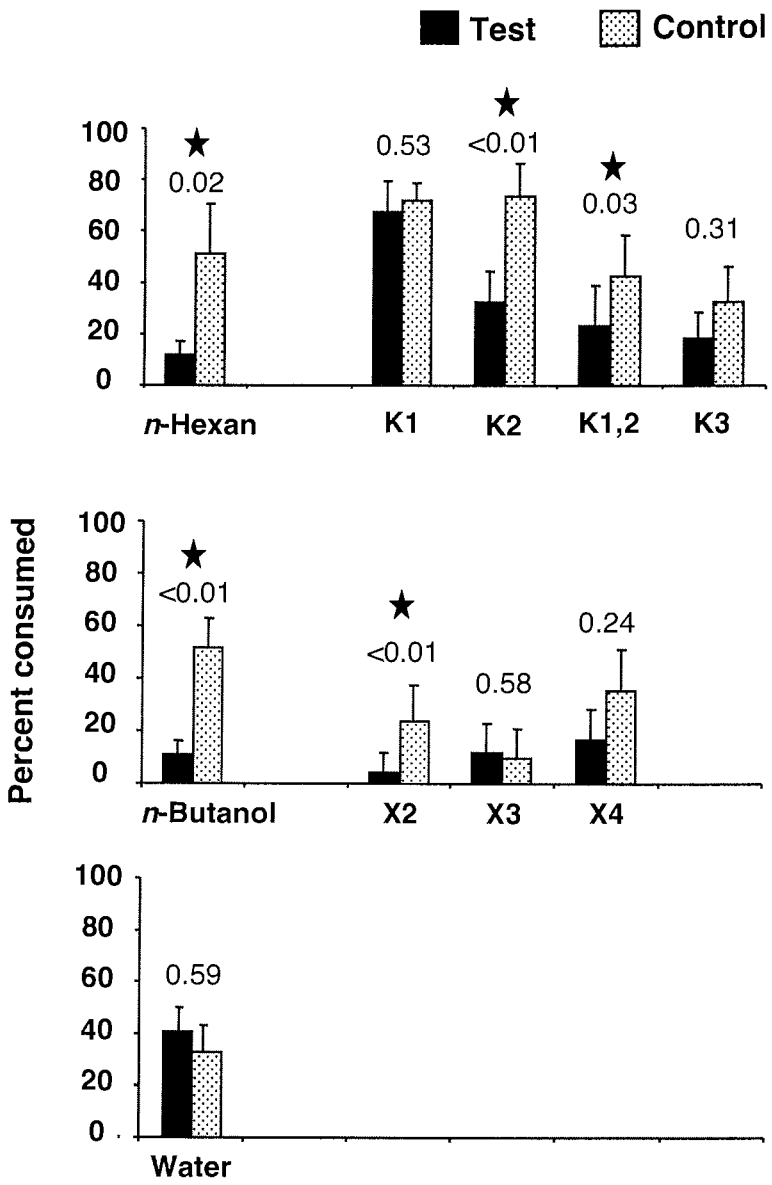

Figure 4: Consumption (mean percentage ± SD) of pellets treated with fractions and isolated compounds of the sponge *Haliclona viscosa* in palatability assays with amphipods Asterisks indicate significant differences (α<0.05, Student's t-test) between consumption of test and control pellets. N=5 for hexane K1; N=7 for hexane K2, N=8 for all other experiments.

Sixteen of the invertebrate species investigated in the amphipod assay were also tested in the feeding experiments with the starfish *Asterias rubens* from the North Sea (Fig. 5). The two nudibranch species, *Dendronotus frondosus* (Ascanius) and *Flabellina salmonacea* (Couthouy) could not be tested due to limited amounts of extract available. Overall, the results of the starfish assay are similar to those of the assays with amphipods. The only extract rejected by the amphipods but consumed by the starfish was the actinian *Hormathia nodosa*. Activity of fractions was not tested in starfish assays because only limited amounts of material were available.

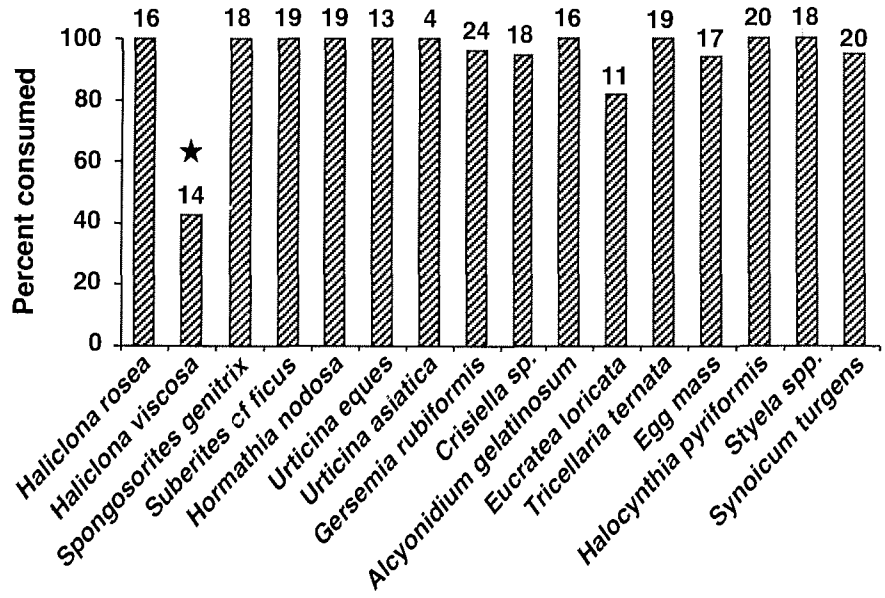

Figure 5: Percent (mean ± SD) of test and control pellets consumed in palatability assays with the starfish *Asterias rubens*. Number of replicates is given above bars. In all experiments 100 % of the control pellets were eaten. Asterisks indicate significant differences (α<0.05, Fisher's Exact test) between consumption of test and control pellets. *Haliclona viscosa* P=0.002, *Eucratea loricata* (Linnaeus) P=0.476, others P=1.

Five of the extracts without significant effect on consumption (*Haliclona rosea* (Bowerbank), *Spongosorites genitrix* (Schmidt), *Dendronotus frondosus*, *Halocynthia pyriformis* (Rathke), *Gersemia rubiformis* (Ehrenberg) were additionally tested in concentrations of two to five times higher than natural,

depending on extract availability. Only the crude extract of the soft coral *Gersemia rubiformis* had an inhibiting effect on amphipod (P=0.009) and starfish (P=0.018) feeding at concentrations five fold higher than natural (Fig. 6).

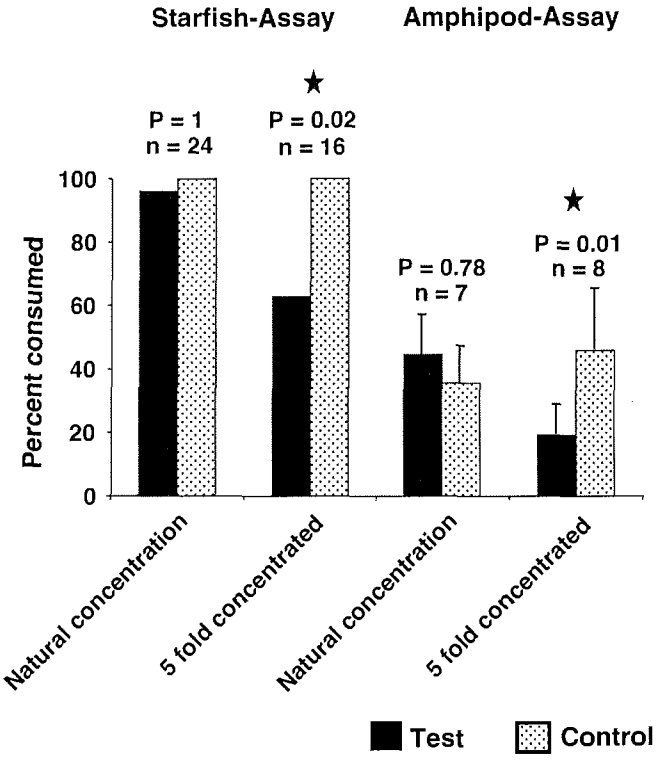

Figure 6: Palatability of crude extract of *Gersemia rubiformis* in feeding assays with starfish and amphipods (mean percentage comsumption ± SD). Extracts were tested in natural concentration and in 5 fold natural concentration. Asterisks indicate significant differences (α<0.05, Fisher's exact test in amphipod assays, Student's t-test in starfish assays) between consumption of test and control pellets. Number of replicates and P values are given above bars.

DISCUSSION

The incidence of chemical defence in marine organisms is suggested to be higher in species from tropical regions compared to species from higher latitudes, due to a lower selective pressure by predation and grazing (Bakus and Green, 1974; Hay and Fenical, 1988; Bolser and Hay, 1996) For Antarctic regions, however, several recent studies have shown that this latitudinal relationship does not hold true (Avila

et al., 2000; Amsler et al., 2001). In Antarctic waters, predation by invertebrates has been found to be a selective pressure for the development of chemical defences (McClintock and Baker, 1997). The latitudinal hypothesis has not been tested yet towards the northern extension of the latitudinal gradient and virtually nothing is known on the occurrence of chemical defences in Arctic waters. Our study is one of the first to address the topic of the abundance and ecological significance of secondary metabolites in invertebrates from northern high latitudes. The incidence of feeding deterrence in invertebrates from Kongsfjord is very low. Only three out of eighteen species exhibited chemical defence against predation at natural or higher concentrations. This suggests a low percentage of chemically defended species compared to tropical (Bakus and Green, 1974; Green, 1977; Pawlik et al., 1995) and Antarctic regions (McClintock and Baker, 1997; Amsler et al., 2001), and it is also in the lower range of that found in temperate waters (*fide* Bakus and Green, 1974). Direct comparisons between the different studies, however, have to be considered cautiously because of the variety of methods used and the different bioassay organisms employed.

Crude extracts of the actinian *Hormathia nodosa* and the sponge *Haliclona viscosa* had a significantly feeding deterrent effect on at least one of the predators used in our assays. Both predators, the starfish and the amphipods, rejected *Haliclona viscosa*. Several species of the genus *Haliclona* from tropical and Antarctic regions are reported to contain biologically active compounds (e.g. Bakus et al., 1994; Harrison, 1999). Many *Haliclona* species are known to be especially rich in sterols (Elenkov et al., 1999) but also terpenes and alkaloids (e.g. Parameswaran et al., 1998). For example, the Antarctic species *Haliclona* dancoi showed feeding deterrent activity against predatory starfish (McClintock, 1987). Interestingly enough, of the two species of the genus *Haliclona* (*H. viscosa*, *H. rosea*) tested in our feeding assays, only the extract of *H. viscosa* showed feeding deterrent activity. Lippert and Iken (in prep.) found tissue of these two species rejected by natural assemblages of predators *in situ* and by the starfish *Asterias rubens* in laboratory assays, indicating that *H. rosea*, although not defended chemically, is likely to be defended against predation by other mechanisms, for example structural elements. Also Waddell and Pawlik (2000) found the Caribbean species *Haliclona hogarthi* to be chemically non-defended

and palatable to various starfish predators, however, in this case whole tissue was readily consumed.

The resistance of cnidarians to predation is often attributed to nematocysts and associated proteinaceous toxins, but is also known to be based on chemical defences (Stachowicz and Lindquist, 2000). We found extracts of the actinian *Hormathia nodosa* to be chemically defended, while extracts of the other two actinians, *Urticina eques* and *U. asiatica*, did not deter amphipod and starfish feeding. Tissue of *U. eques* was found to be significantly unpalatable to natural assemblages of predators in the field and to the starfish *Asterias rubens* (Lippert and Iken, in prep.), indicating that nematocysts may have a deterrent effect since chemical defence does not seem to be responsible for the rejection by predators.
All feeding bioassays were performed with extracts at estimated natural concentrations (see Table 1) in order to assay extracts at ecologically relevant dosages. However, bulk estimations from extract yield per gram dry weight extracted tissue may underestimate natural extract concentrations. Defensive substances are often not distributed evenly throughout the body tissue of an organism but instead are concentrated in tissues which have a higher likelihood to be encountered by predators (Schupp et al., 1999) or in biologically valuable parts like reproductive regions (Pawlik et al., 1988). For example, Tipton, Baker and McClintock (unpublished data - as cited in Amsler et al., 2001) and Becerro et al. (1997) found active compounds to accumulate in the outer layers of Antarctic and Mediterranean sponges, respectively. Considering this, secondary metabolite concentrations may be higher than estimated in some species and thus also the number of chemically defended species may be higher. We therefore repeated tests of five species (the sponges *Haliclona rosea* and *Spongosorites genitrix*, the octocoral *Gersemia rubiformis*, the nudibranch *Dendronotus frondosus* and the ascidian *Halocynthia pyriformis*) at higher concentrations of crude extracts. We selected for the sponges, because species of this taxonomic group are often known to be rich in secondary metabolites, and often possess feeding deterrent compounds (e.g. McClintock, 1987; Wadell and Pawlik, 2000). Also many nudibranch species have developed chemical defences (Avila, 1995). *Gersemia rubiformis* was chosen because a close relative from Antarctica, *Gersemia antarctica*, is known to be chemically defended against predatory starfish (Slattery and McClintock, 1995). *Halocynthia pyriformis* was conspicuous from our own

underwater observations in not having any fouling organisms on its surface. Only the crude extract of *Gersemia rubiformis* significantly deterred amphipod feeding at five fold natural concentration while for the four other extracts the increase in concentration did not show any effect. Consequently the experiments with higher concentrated crude extracts mainly confirm the results of feeding assays with estimated natural concentrations indicating that feeding deterrence was not significantly underestimated in the present study.

Interestingly, we found that extracts of several invertebrate species were significantly preferred over the control food in amphipod assays, while others were consumed at the same level as the controls, and only two extracts were significantly rejected. Six of the investigated species had a significantly stimulating effect on amphipod feeding in the two-choice feeding experiments, indicating that crude extracts may contain compounds, such as fatty acids, steroids or other secondary metabolites, that make test pellets more attractive to amphipods.

We have tested palatability of organic crude extracts of invertebrate species from Kongsfjord, Spitsbergen, to two predators, the amphipod *Anonyx nugax* from Kongsfjord, and the starfish *Asterias rubens* from the North Sea. Overall, we found that amphipods and starfish show similar feeding preferences. Crude extracts from only two out of 18 species tested deterred amphipod feeding significantly, one of which was also significantly deterrent in the starfish assay. The only difference between both assays was found for the actinian *Hormathia nodosa*, which was readily eaten by the starfish while it was significantly unpalatable to the amphipods. The observed differences in palatability of extracts in starfish and in amphipod assays may be due to a different experimental design: The amphipod feeding assays were designed as a two-choice experiment while the starfish had a single choice. Feeding preferences of predators can vary depending on the number of offered food choices (Lubchenco and Gaines, 1981). In a two-choice feeding experiment slight feeding deterrence of an extract could be sufficient to make predators select for the more palatable control food, while in a single-choice experiment a slightly feeding deterrent extract may still be accepted as a suitable food.

In the first part of this study we showed that crude extracts of *Hormathia nodosa* and *Haliclona viscosa* were deterrent at their natural concentrations towards general consumers. In the second part of the study, deterrent crude extracts were further partitioned. The *n*-BuOH fraction of *Hormathia nodosa* had a significant inhibiting effect on amphipod feeding, suggesting the involvement of more polar compounds in feeding deterrence, but we have yet not been able to isolate active metabolites from this fraction. Fractions as well as pure compounds of the active EtOAc/*n*-BuOH and *n*-hexane fractions of *Haliclona viscosa* were tested for their palatability to amphipods. One compound from the *n*-hexane (K2) and one compound from the EtOAc/*n*-BuOH fraction (X2) significantly deterred amphipod feeding. Their occurrence in different fractions shows that they are two distinct compounds which both exhibit feeding deterrence independently. The activity of several compounds characterises *Haliclona viscosa* as a chemically rich species compared to other species from Kongsfjord. Although K2 and X2 were active in feeding deterrence, other experiments testing microbial activity of both compounds against sympatric bacteria suggest a certain specificity (Lippert et al., in press).

Sponges of the order Haplosclerida (class Demospongiae, subclass Ceractionmorpha) are known to contain various 3-alkylpyridines or their reduction products (especially the genera *Haliclona*, *Xestospongia* and *Amphimedon*). These are structurally diverse, but related, polycyclic alkaloids with two heterocyclic nitrogens and no aliphatic methyl groups. Several of these compounds are known to show strong cytotoxic activity (Andersen et al., 1996). The structural elucidation of the three compounds isolated from the EtOAc/*n*-BuOH fraction of *H. viscosa* in the present study showed that X2 is related to the alkaloid cyclostelletamine C described from *Stelletta maxima* (Fusetani et al., 1994) while X3 and X4 are related to the alkaloid haliclamine A from *Haliclona* sp. known from Fusetani et al. (1989). The main problem in the structure elucidation of X3 and X4 was that the two heterocycles are connected by saturated alkyl chains. The chemical structure of the active compound K2 has yet to be identified.

Our results from Kongsfjord confirm the inverse relationship between latitude and incidence of chemical defence. As stated by Thorson (1957) and Gulliksen (1979) predation is low in the Arctic and may thus not be a strong selective force

to drive the evolution of chemical defence in Arctic marine systems. We suggest that this is likely to be true for the Kongsfjord where we observed only low diversity and abundance of predators, mainly amphipods and some starfish and gastropods (Lippert and Iken, in prep.). However, Kongsfjord, although located at high latitude, is under the influence of the relatively warm West Spitsbergen Current carrying water from the northernmost extension of the North Atlantic Current (Svendsen et al., 2002), and therefore has to be regarded as a sub-Arctic rather than a high Arctic fjord (Hop et al., 2002). But even more importantly, Kongsfjord is strongly influenced by glacial activity. High sedimentation rates, freshwater influence and ice scouring are known to have a considerable structuring effect on the fauna especially of inner fjord locations (Wlodarska-Kowalczuk et al., 1998). Therefore, biological dynamics in Kongsfjord might be representative for western Spitsbergen fjord environments (Hop et al. 2002), but conditions may be very different at other locations in high northern latitudes that are more under the influence of Arctic water masses, such as the northern and the eastern shores of Spitsbergen (Wlodarska-Kowaltczuk, et al. 1998). Although the presented findings from Kongsfjord support our initial assumption of a low incidence of chemical defence in Arctic waters , it has to be kept in mind that this is a single location and may not be representative for other Arctic regions. Therefore, more chemical ecology studies at different sites in the high northern hemisphere are necessary to draw general conclusions on the significance of chemical defences in Arctic waters.

ACKNOWLEDGEMENTS

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PUBLICATION III

ANTIMICROBIAL ACTIVITY IN SUB-ARCTIC MARINE INVERTEBRATES

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ABSTRACT

In the marine environment any living or non-living surface is exposed to bacterial colonisation. Many invertebrate species in temperate, tropical and Antarctic regions have demonstrated chemical defenses against the formation of microbial films. In the present study the antimicrobial activity of sub-Arctic invertebrates was investigated for the first time. Crude extracts of abundant invertebrates belonging to several taxonomic groups were tested for their inhibitory effects on the growth of five sympatric phylogenetically diverse bacterial strains. Six out of 18 (33%) crude extracts inhibited bacterial growth at natural extract concentrations. The crude extract of the sponge *Haliclona viscosa* inhibited growth of all five bacterial strains, suggesting the presence of metabolites with broad-spectrum activity. Three active compounds were isolated from *H. viscosa* having antibacterial properties similar to those of the crude extract. Our data indicate that antibacterial secondary metabolites are present in sub-Arctic marine invertebrates but are less abundant than in temperate, tropical, or Antarctic species.

INTRODUCTION

The flora and fauna in the marine environment is virtually bathed in a ‚microbial soup' (Jenkins et al. 1998), and any solid living or non-living surface is exposed to colonization by bacteria. Formation of so-called microbial biofilms is initiated by the adsorption of macromolecules to a surface immediately after contact with seawater, followed by adsorption and adhesion of bacteria (Davis et al. 1989; Wahl 1989). Bacterial colonization and surface conditioning have been considered to be the first stages of surface fouling, promoting subsequent settlement of unicellular and multicellular eukaryotic organisms (Wahl 1989). Filter-feeding animals, such as sponges, that feed on bacteria (Berquist 1978) concentrate microorganisms from the water column during the feeding process and are thus

additionally exposed to high quantities of microbes including potential pathogens. However, the incidence of infection appears to be relatively low in nature, considering the permanent presence of all types of bacteria (Jenkins 1998). Bacteria often do not colonize marine organisms uniformly, since microbial colonization can be reduced by mechanical processes like tissue or mucus sloughing (Krupp 1985; Barthel and Wolfrath 1989), by physical properties like surface tension (Becker and Wahl 1991), by surface acidity (Hirose et al. 2001) and as well by secondary metabolites (McCaffrey and Endean 1985; Wahl et al. 1994; Henrikson and Pawlik 1995; Newbold et al. 1999).

Studies on the chemical antibacterial properties of invertebrates cover a wide range of taxonomic groups and geographic regions. Investigations addressing geographical variations often propose chemical defenses to be increased in tropical organisms compared to temperate species (Hay and Fenical 1988; Bolser and Hay 1996; Bakus 1974; Bakus and Green 1974; Green 1977). However, McCaffrey and Endean (1985) found antimicrobial activity in tropical and sub-tropical sponges from the Pacific to be similar to that in organisms from temperate regions. More recently, some investigations included chemical defenses against surface bacteria in polar waters (McClintock 1987; Slattery et al. 1995). McClintock and Gauthier (1992) found antimicrobial properties in Antarctic sponges to be widespread although the activity was generally weaker compared to temperate and tropical species, indicating a latitudinal decline of activity. So far, little information on latitudinal variation in antimicrobial chemical defenses of marine invertebrates is available from Arctic waters.

It could be hypothesized that organisms would invest more resources into antimicrobial defense when the threat of bacterial colonization is higher. Although bacterial numbers alone do not necessarily reflect the selective pressure exerted on marine invertebrates, it could be one indication of the potential of detrimental surface colonization or infection. Total bacterial numbers in cold waters are proposed to be generally decreased compared to lower latitudes. In the Beaufort Sea, for example, 0.2-0.9 x 10^6 bacteria per ml are found (Atlas and Morita 1986), similar to <0.37 x 10^6 ml^{-1} in Antarctic waters (Zdanowski 1995). Bacterial abundance is higher by almost an order of magnitude in the Baltic Sea (10.9 x 10^6 ml^{-1}, Lignell et al. 1992), in the Atlantic (2.6 x 10^6 ml^{-1}, Hanson et al. 1988), and the Pacific Ocean (<5 x 10^6 ml^{-1}, McManus and Peterson 1988). Locally, however,

bacterial numbers can be increased up to 8 x 10^6 ml^1 at Arctic inner fjord locations, due to a high amount of particulate organic carbon at glacial meltwater outflows (Jankowska and Wlodarska-Kowalczuk, in prep.).

The aim of the present study was to assess, for the first time, the chemical antimicrobial properties of various invertebrates in a fjord at Spitsbergen. Effects of crude extracts, fractions and pure compounds of high latitude invertebrates on sympatric microorganisms were tested. We screened a broad spectrum of abundant marine invertebrate taxa from Kongsfjord, including sponges, actinians, soft corals, molluscs, bryozoans, and ascidians for antibacterial activity.

MATERIALS AND METHODS

STUDY SITE

Our study was performed at Kongsfjord, situated at the northwestern coast of Spitsbergen (79°N, 12°E). A comprehensive overview on the present biological and physical knowledge of the fjord is provided in two recent reviews (Hop et al. 2002; Svendsen et al. 2002). The fjord extends 26 km land inwards with a width of 3 to 8 km and a maximum depth of about 400 m. Despite the high latitudinal location the fjord has rather sub-Arctic than Arctic properties, due to the strong influence of relatively warm Atlantic water masses at the northwestern coast of Spitsbergen (Svendsen et al. 2002). The summer season is characterized by considerable glacial activity with four glaciers and several river run-offs adding high loads of terrestrial sediments and freshwater to the fjord (Elverhoi et al. 1983; Weslawski et al. 1995). The annual mean water temperature is slightly above 0°C (Ito and Kudoh 1997), however, in summer maximum temperatures of about 6°C at the surface and of about 4°C at 20 m depth were measured (Hanelt et al. 2001). Invertebrates were collected at four different sites in the fjord. While sites 1, 2 and 3 (Hansneset, a cave close to Hansneset, and Kongsfjordneset, Fig. 1) are dominated by hard bottom communities, site 4 (Prinz Heinrich Islands, Fig. 1) is characterized by soft sediments with single stones and boulders, forming patches of hard substrate habitats.

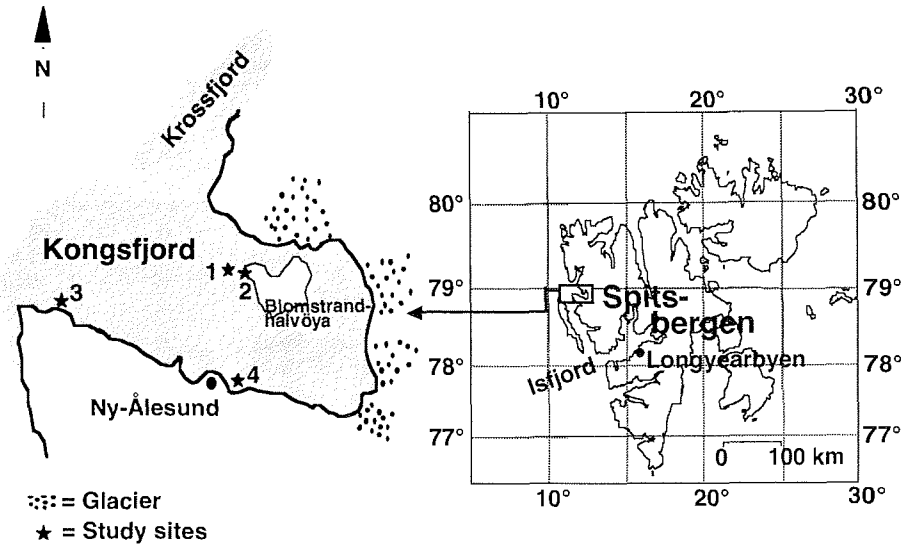

Figure 1: General view of Spitsbergen, showing Kongsfjord and the study sites. Asterisks and numbers indicate the different sampling sites (1-4): 1 – Hansneset; 2 – Cave; 3 – Kongsfjordneset, 4 – Prinz Heinrich Islands.

SAMPLING

Seventeen species of sessile or slow moving invertebrates which were abundant at the different sampling sites, and the egg mass of a gastropod were collected by SCUBA diving in the summers of 1999, 2000 and 2001. Samples were immediately brought to the laboratory and epibiontic organisms, when present, were removed manually from the surfaces. After gently blotting with tissue paper to remove extracellular water, samples of each species were weighed, shock frozen in liquid nitrogen and lyophilized. The freeze-dried tissue was weighed again and stored at –28°C until extraction. For later species identification voucher specimens were preserved in a 5% (v/v) formaldehyde seawater solution. Information on the taxa studied, the respective collection sites and depth of sampling are given in Table 1. Taxonomic identification of the ascidian *Styela* spp. showed that the collected material consisted of a mixture of the two morphologically similar species *Styela rustica* and *Styela gelatinosa*.

Table 1: Investigated invertebrate species, sampling site and water depth of sampling, extraction method and estimated natural concentration of crude extracts. Extrtaction method A indicates extraction in 1:1 MeOH/DCM, method B means subsequent extraction in 100% MeOH, 1:1 MeOH/DCM, 100% DCM. Natural concentrations are given in mg extract / g WW.

	Taxa	Collection site	Depth of collection [m]	Extraction method	Natural concentration [mg/WW]
Porifera	*Haliclona viscosa*	Hansneset	15-25	B	36.3
	Haliclona rosea	Hansneset	10-25	B	18.8
	Suberites ficus	Cave	4-5	A	46.3
	Spongosorites genitrix	Cave	4-5	A	38.6
Cnidaria	*Hormathia nodosa*	Hansneset	10-25	A	34.9
	Urticina eques	Kongsfjordneset	10-25	A	27.7
	Urticina asiatica	Kongsfjordneset	10-25	A	19.3
	Gersemia rubiformis	Cave	2-4	A	44.1
Bryozoa	*Tricellaria ternata*	Hansneset	1-10	A	38.2
	Eucratea loricata	Hansneset	1-6	B	26.2
	Crisiella sp.	Hansneset	1-6	A	43.8
	Alcyonidium gelatinosum	Hansneset	1-8	A	29.6
Gastropoda	*Natica* sp. (egg mass)	various sites	4-30	A	10.2
Nudribranchia	*Dendronotus frondosus*	various sites	1-25	A	39.5
	Flabellina salmonacea	various sites	1-25	A	30.3
Ascidiacea	*Halocynthia pyriformis.*	Hansneset	5-25	B	25.8
	Styela spp. (*S.gelatinosa, S. rustica*)	Prinz Heinrich Islands	15-30	B	45.4
	Synoicum turgens	Hansneset	1-7	A	13.1

EXTRACTION PROCEDURE

A known mass of freeze-dried tissue from each species was ground with mortar and pestle and subsequently extracted in 100% methanol, 1:1 (v/v) methanol:dichloromethane, and 100% dichloromethane, or repeatedly in 1:1 (v/v) methanol:dichloromethane under permanent stirring on a magnetic stirrer at room temperature. The partial extracts were combined (see Table 1 for details) and filtered to remove particles, followed by concentration under reduced pressure using a rotary evaporator (40°C). Crude extracts were transferred into pre-weighted vials, evaporated to dryness under nitrogen or vacuum, and weighed. The extract yield per g FW (Table 1) is referred to as the natural concentration. This is the most widely used approach but it may not always reflect the actual concentration at an organism's surface in nature (Pawlik et al. 1988; Becerro et al. 1997; Schupp et al. 1999), and slight over- as well as underestimations may be possible. Attempts to elucidate actual surface concentrations of antifouling

compounds have only recently begun for macroalgae (De Nys et al. 1998) and more research is necessary to develop this methodology further. To obtain sufficient mass of crude extracts we used pooled samples of several individuals. All extracts were stored at –28°C until use in antibacterial experiments.

Crude extracts of *Haliclona viscosa* and *Halocynthia pyriformis*, both exhibiting antibacterial activity, were further partitioned with *n*-hexane. The aqueous phase was dried and re-dissolved in water, followed by subsequently partitioning with ethyl acetate (EtOAc) and butanol (*n*-BuOH). The EtOAc and *n*-BuOH fractions of *H. viscosa* were combined after HPLC analysis revealed no differences in their chemical composition. The remaining water fractions of both species were also subjected to bioassays. The active EtOAc/*n*-BuOH fractions of *H. viscosa* were further purified with low-pressure liquid chromatography (silica gel, hexane:EtOAc 6.5:3.5) and preparative RP-HPLC using a Kromasil RP 18 column (4.6 x 250 mm, 5 µm particle size), respectively.

BACTERIAL ISOLATION AND SEQUENCE ANALYSIS

Bacterial strains from the vicinity of the investigated invertebrates were isolated from stones, sediment and seawater from about 12 m water depth at site 1 (Fig. 1.) These samples were collected using sterile plastic bags and were immediately transferred to the laboratory. Bacteria were obtained from stones by swabbing the surface with a sterile cotton tip followed by inoculation of agar plates. Sediments were suspended in ten times volume of sterile seawater, allowed to settle and 100 µl of the supernatant were used for inoculation of agar plates using a sterile glass rod. From seawater samples 100 µl aliquots were directly spread on agar plates. We used marine agar medium after Zobell (1941) in two different nutritional concentrations including a low-nutrient agar that may reflect better the nutrients available to marine bacteria in their natural environment (Zobell I: 94% sea water, 5% peptone, 0.9% yeast extract, 0.1% $FePO_4$; Zobell II: 99.4% sea water, 0.5% peptone, 0.1% yeast extract, 0.01% $FePO_4$). Bacteria were incubated at 4°C, which approximately equals seawater temperature in the Kongsfjord during summer. Numerous pure cultures were established from the different substrates.

From all cultures five bacterial strains (Table 2) were chosen and identified by sequence analysis of 16S rDNA. Nucleic acids were extracted from the isolated

cultures with the DNeasy Tissue Kit (Qiagen, Hilden, Germany). From template DNAs (approx. 100 ng) 16S rDNAs were amplified by hotstart PCR (Saiki et al. 1988) with an automated thermal cycler (Eppendorf, Germany) using the bacterial-specific primers *8f* (5'-AGA GTT TGA TCM TGG C-3') (Giovannoni 1991) and *1492r* (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Lane 1991) as follows: 50 pmol of each primer, 2.5 μmol of each deoxyribonucleoside triphosphate, 1 x PCR buffer, 40 μl Taq Master enhancer (pre-heated, Eppendorf) were adjusted to 100 μl with sterile water. The addition of Taq Master to reaction mixture improved the success of the PCRs. Master Taq (1 U, Eppendorf) was added to the reaction mixture at 60°C after an initial 5 min denaturation step at 95°C. The cycles were as follows: 30 cycles at 95°C for 1 min, 45°C for 1 min, and 72°C for 3 min; and a final elongation step at 72°C for 10 min. PCR products were purified using the QIAquick Purification Kit (Qiagen). 16S rDNAs were sequenced with an ABI PRISM 3700 capillary sequencer (Applied Biosystems, Inc., Foster City, CA) using AmpliTaq DNA Polymerase and 16S rDNA specific primers. Sequence data were analyzed with the ARB software package (http://www.mikro.biologie.tu-muenchen.de) and a phylogenetic tree was reconstructed using maximum-likelihood analyses. Only 16S rDNA sequences containing at least 1400 bases were used for tree construction. Filters for phylogenetic subdivisions and/or groups which considered only 50% conserved regions were applied to exclude highly variable positions.

The 16S rRNA sequences generated in this study were deposited into the GenBank database (Benson et al. 1999) under the following accession numbers: isolate A, AY198113; isolate B, AY198114; isolate C, AY198115; isolate D, AY198116; and isolate E, AY198117.

ANTIMICROBIAL ASSAY

The agar disc-diffusion assay (Acar 1980) was used to test for antibacterial activity of extracts of seventeen invertebrate species and the egg mass of a gastropod (Table 1) against the five sympatric bacterial strains. To guarantee high cell densities, bacterial strains were grown in liquid medium (99.4% seawater, 0.5% peptone, 0.1% yeast extract, 0.001% $FePO_4$) at 4°C for at least 7 days prior to the experiments. Inocula of 200μl of each strain were then spread on separate agar

plates (99.4% seawater, 0.5% peptone, 0.1% yeast extract, 0.001% $FePO_4$) using a sterile glass rod to provide a uniform film of the test bacteria.

Crude extracts and fractions were dissolved in aliquots of the extraction solvent to give natural concentrations (Table 1). Pure compounds were brought up in concentrations of 5 mg/ml. Since none of the three compounds isolated from the EtOAc/*n*-BuOH fraction of the sponge *Haliclona viscosa* (X2, X3, X4) exhibited antibacterial effects comparable in strength to the effects caused by the EtOAc/*n*-BuOH fraction, these compounds were recombined to test for synergistic effects. Compounds for recombination were used in the respective proportions they contributed to the total yield of the EtOAc/*n*-BuOH fraction.

We applied 10 µl of crude extract, fraction or pure compound onto each side of a sterile paper disc (Ø 6 mm, Whatman). The volume added represents the approximate volumetric capacity of the discs. Discs were then placed in a previously sterilized (160°C) drying oven at 30°C and solvents were allowed to evaporate. Control discs were prepared in the same manner with solvent only. Up to six extract discs and one solvent control disc per plate were placed on the surface of agar plates previously seeded with individual bacterial strains. According to extract availability one to five replicates per extract were tested. The radius of the inhibition zone (without disc) was measured to the nearest 0.5 mm following incubation at 4°C over five days. Solvent control discs were never observed to inhibit bacterial growth. Since bacterial films of strains B and C were still very transparent after five days and zones of inhibition were difficult to distinguish, we measured inhibition zones again after six additional days for all five strains. Only minor differences in size of inhibition zones were found between measurements after five and eleven days. Results were categorized as no effect (0), weak inhibition (0 to 1 mm), moderate inhibition (>1 to 3 mm), strong inhibition (>3 to 7 mm) and very strong inhibition (>7–15 mm). Due to the quantity of extract available the number of replicates varied between one and six.

RESULTS

IDENTIFICATION OF BACTERIAL STRAINS

Bacterial isolates were phylogenetically diverse and clustered within clades of previously cultured bacteria from polar oceans or sea ice (Fig. 2). Isolates A, C, and D were most closely related to *Psychrobacter* spp., *Colwellia* spp., and *Pseudoalteromonas* spp., respectively, within the γ-proteobacteria. Isolate E, within the *Cytophaga/Flavobacterium* (C/F) group, associated with *Polaribacter* spp. and isolate B, a Gram-positive bacterium, associated with *Planococcus* spp.

Table 2: Bacterial strains isolated from the vicinity of the investigated invertebrates.

Bacterial strain and accession number	Isolated from	Medium	Gram staining
A (AY198113)	water column	Zobell I	negative
B (AY198114)	stone	Zobell I	positive
C (AY198115)	stone	Zobell I	negative
D (AY198116)	sediment	Zobell I	negative
E (AY198117)	water column	Zobell II	negative

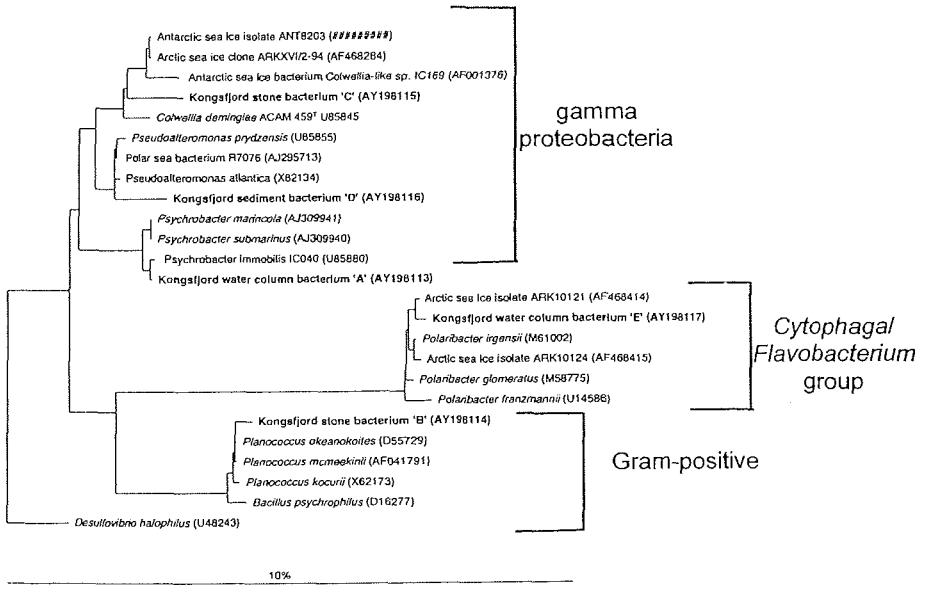

Figure 2: Phylogenetic tree based upon maximum likelihood (FastDNAml) analysis. Outgroup is *Desulfovibrio halophilus* (U48243). The scale bar indicates 10% estimated sequence divergence. The lengths of vertical lines are not significant. Isolated strains 'A', 'B', 'C', 'D', 'E' are in bold type.

ANTIBACTERIAL TESTS

Table 3: Inhibition of bacterial growth by crude extracts of 18 invertebrate species. Radius of inhibition zone: 0 = no effect; 0-1mm = X (weak inhibition); >1-3 mm = XX (moderate inhibition); >3-7 mm = XXX (strong inhibition); >7-15 mm = XXXX (very strong inhibition).

Species	Replicate	Bacterial strain					∑ strains inhibited
		A	B	C	D	E	
Haliclona rosea	1	0	0	0	n.d.	n.d.	-
	2	0	0	0	0	0	0
Haliclona viscosa	1	XXX	XXXX	XXXX	XXX	XXXX	5
Spongosorites genitrix	1	0	0	0	0	0	0
Suberites ficus	1	0	0	0	n.d.	n.d.	-
	2	0	0	0	0	0	0
Hormathia nodosa	1	0	0	0	0	0	0
Urticina eques	1	0	0	0	0	0	0
Urticina asiatica	1	0	0	0	0	0	0
Gersemia rubiformis	1	0	X	XXX	0	0	2
5-fold	1	0	XXX	XXXX	0	0	2
Tricellaria ternata	1	0	0	0	n.d.	n.d.	-
	2	0	0	0	0	0	0
Eucratea loricata	1	0	0	0	0	0	0
Alcyonidium gelatinosum	1	0	X	X	n.d.	n.d.	-
	2	0	XXX	X	0	0	2
Crisiella sp.	1	0	XX	0	0	0	1
Dendronotus frondosus	1	0	0	0	0	0	0
Flabellina salmonacea	1	0	X	X	0	0	2
Synoicum turgens	1	0	0	0	0	0	0
Halocynthia pyriformis	1	0	0	X	0	0	1
5-fold	1	0	XX	n.d.	0	XX	-
Styela spp.	1	0	0	0	0	0	0
5-fold	1	0	X	0	0	0	1
Egg mass of *Natica* sp.	1	0	0	0	0	0	0
∑extracts with inhibiting effect		1	7	5	1	2	

Results of antimicrobial experiments testing crude extracts of 17 invertebrate species and of the egg mass of a gastropod against five strains of sympatric bacteria are shown in Table 3. Antibacterial effects differed with respect to extracts

and bacterial strains. Six out of 18 extracts inhibited growth of at least one bacterial strain at natural extract concentrations. The extract of the sponge *Haliclona viscosa* had the strongest antimicrobial activity in terms of the number of strains inhibited and the radius of the inhibition zones. Only *Haliclona viscosa* inhibited the growth of all five test bacteria, while the soft coral *Gersemia* rubiformis, the bryozoan *Alcyonidium gelatinosum* and the nudibranch *Flabellina salmonacea* inhibited two strains, the bryozoan *Crisiella* sp. and the ascidian *Halocynthia pyriformis* both inhibited one strain. The two bacterial isolates most frequently inhibited were strains B and C.

Crude extracts of the soft coral *Gersemia rubiformis* and the ascidians *Halocynthia pyriformis* and *Styela* spp. were additionally tested at five-fold natural concentrations (Table 3). This increase in concentration caused an increase in inhibition zones of strain B and C in response to the extract of *Gersemia rubiformis*, as well as an inhibition of two additional strains (B and E) by the extract of *Halocynthia pyriformis*. The extract of *Styela* sp. did not have any effect on bacterial growth at natural concentration, but had slightly inhibiting effects on strain B at five-fold natural concentration.

Active crude extracts of *Haliclona viscosa* and *Halocynthia pyriformis* were further partitioned and antimicrobial activity of fractions was tested in the agar disc-diffusion assay. The crude extract of *H. viscosa* was separated into three fractions, yielding an *n*-hexane, an EtOAc/*n*-BuOH and an aqueous remain fraction. EtOAc, *n*-BuOH, *n*-hexane and water fractions were obtained from the crude extract of *H. pyriformis*. The EtOAc/*n*-BuOH soluble compounds from *H. viscosa* and the EtOAc fraction from *H. pyriformis* inhibited bacterial growth, whereas all other fractions of both species had no effect (Table 4). Antibacterial activity of the EtOAc fraction of *H. pyriformis* was very low at natural concentration (< 1 mm, strain B) but increased at five-fold natural concentration (1-2 mm, strain B and C). Growth of strain E was not affected by the EtOAc fraction, although the crude extract showed weak growth inhibition of this bacterial strain.

Table 4: Inhibition of bacterial growth by crude extract, fractions and pure compounds of the sponge *Haliclona viscosa* and the ascidian *Halocynthia pyriformis*. Radius of inhibition zone: 0 = no effect; X = 0-1mm (weak inhibition); XX = >1-3 mm (moderate inhibition); XXX = >3-7 mm (strong inhibition); XXXX = >7-15 mm (very strong inhibition). The number of replicates is given in paranthesis.

Extract	Bacterial strain					∑ strains inhibited
	A	B	C	D	E	
• ***Haliclona viscosa***						
Crude extract	XXX (4)	XXXX (1)	XXXX (1)	XXX (4)	XXX (4)	5
n-hexan	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0
n-BuOH/EtOHc	XX (5)	XXX (2)	XX (1)	XXX (6)	XXX (5)	5
water	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0
X2	XX (3)	XX (1)	0 (1)	XX (3)	XXX (3)	4
X3	0 (3)	XXXX (1)	0 (1)	0 (4)	XXX (3)	2
X4	0 (3)	XXXX (1)	X (1)	0 (4)	XXX (3)	3
X2-X3-X4	0 (3)	n.d.	n.d.	0 (3)	XXX (2)	-
X2-X3	X (2)	n.d.	n.d.	0 (2)	XXX (2)	-
X2-X4	X (2)	n.d.	n.d.	0 (2)	XXX (2)	-
X3-X4	0 (2)	n.d.	n.d.	0 (2)	X (2)	-
• ***Halocynthia pyriformis***						
natural concentration						
Crude extract	0 (1)	0 (1)	X (1)	0 (1)	0 (1)	1
n-hexane	n.d.	0 (1)	0 (1)	0 (1)	0 (1)	0
n-BuOH	n.d.	0 (1)	0 (1)	0 (1)	0 (1)	0
EtOAc	n.d.	X (1)	0 (1)	0 (1)	0 (1)	1
Water	n.d.	0 (1)	0 (1)	0 (1)	0 (1)	0
5-fold natural concentration						
Crude extract	0 (1)	XX (1)	n.d.	0 (1)	XX (1)	-
n-hexane	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0
n-BuOH	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0
EtOAc	0 (1)	XX (1)	X (1)	0 (1)	0 (1)	2
Water	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0

Further purification of the active *n*-BuOH/EtOAc fraction of *H. viscosa* resulted in three pure compounds X2, X3 and X4. Structural analysis of these compounds is still ongoing but preliminary results show that they are related to the alkaloids cyclostelletamine (Fusetani et al. 1994) and haliclamine (Fusetani et al. 1989) (Volk et al. in prep.). Results of all *H. viscosa* fractions and compounds are given in Table 4. Inhibition of bacterial growth by the crude extract was stronger (five strains, strong to very strong inhibition) than by the *n*-BuOH/EtOAc-fraction or by the pure compounds. The *n*-BuOH/EtOAc fraction inhibited growth of all bacterial strains but effects were weaker (moderate to strong) than in crude

extract. Purified compounds X3 and X4 exhibited strong to very strong inhibition of test strains B and E while there was no effect on strains A and D, and only a weak effect of X4 on strain C (< 1 mm) (Table 4). Compound X2 had a moderate inhibitory effect on strains A, B and D and a strong effect on strain E, but did not affect strain C. Since none of the effects caused by the individual compounds, X2, X3 and X4, corresponded exactly with the antibacterial effects caused by the crude extract or the *n*-BuOH/EtOAc fraction, we also conducted experiments with all possible recombinations of the three compounds, but did not find any synergistic or additive effects (Table 4).

DISCUSSION

BACTERIAL TEST STRAINS

The bacteria isolated from Kongsfjord and employed in the agar disc-diffusion assays were typical cultivable members of the marine microbial community in cold seas. Members of the genus *Polaribacter* (isolate E) are ubiquitous in polar oceans (Gosink et al. 1998) and several of the isolates (A, B, D) are representative of species associated with surfaces in the marine environment. *Psychrobacter* spp. and *Pseudoalteromonas* spp. as well as Gram-positive bacteria have been found to be associated, for example, with bryozoans in the North Sea (Pukall et al. 2001). The five bacterial strains used here in antibacterial assays thus represent a diverse array of marine bacteria (Fig. 2). They have the potential to come into contact with the invertebrates tested, although we do not know if they would affect these invertebrates positively or negatively. As discussed by Jenkins et al. (1998) ecologically sensible bioassays have to involve the use of microorganisms that interact with the investigated organism in nature. It has been shown that antibacterial activity against non-marine pathogens does not necessarily indicate similar activity against marine bacteria (Bergquist and Bedford 1978; Thompson et al. 1985). Bacterial strains in the present study were isolated from non-living, biologically inert substrates, since bacteria associated with invertebrates may have developed resistance to host metabolites as a result of co-evolution. Kelman et al. (1998) found no activity of coral crude extracts against bacteria isolated from coral

tissue and surface mucus, while, on the other hand, two bacterial strains isolated from sponge tissue were the most sensitive test strains among six genera of marine bacteria against sponge extracts (Newbold et al. 1999). Therefore, the use of bacteria isolated from the same organism that is tested for antibacterial activity may lead to over- or underestimation of the antibiotic potential.

Among the five bacterial strains used in the present study, strains B (Gram-positive) and C (Gram-negative) were most frequently inhibited. Similar to results from Jensen et al. (1996) who found indication that fast- and slow-growing bacteria had different susceptibility to antibiotic extracts, these two obviously sensitive strains B and C were growing slower compared to the other three bacterial strains.

ANTIMICROBIAL ACTIVITY

Knowledge on the antimicrobial activity in marine invertebrates is not distributed evenly among the taxonomic groups, making geographical comparisons within taxa more difficult. While extensive literature exists on sponges (Burkholder and Ruetzler 1969; Newbold et al. 1999) and gorgonian corals (Kim 1994; Jensen et al. 1996), little is known about bryozoans (Walls et al. 1993), and even less about actinians. However, results from the present study indicate that only a small portion of Arctic invertebrates is chemically defended against bacteria compared to species from lower latitudes and also from Antarctica. McClintock and Gauthier (1992), for example, found 64% of 17 Antarctic sponges to be at least weakly active against marine bacteria. In New Zealand, 76% of 30 sponges inhibited growth of marine bacteria (Bergquist and Bedford 1978), 48% of 33 Carribean sponges had antimicrobial activity (Newbold et al. 1999), and 32% of 28 Mediterranean sponges tested against marine bacteria and yeasts were active (Amade et al. 1987). Of the 18 crude extracts tested in this study, six (33%) caused noticeable inhibition of growth of at least one of the five bacterial strains at natural extract concentration. However, we tested several different taxonomic groups and only four of the extracts originated from sponges. Of these sponges one (25%) showed antimicrobial activity.

In the literature, there has been considerable discussion about which size of inhibition zone reliably indicates antibiotic activity. For example, Bergquist and Bedford (1978) ignored all zones of inhibition less than 1 mm, and Jensen et al.

(1996) regarded extracts to possess antibacterial activity only if at least two replicates produced zones >5 mm. Other authors have considered any zone of growth inhibition, independent of its size, as an indication of antimicrobial activity (Thompson et al. 1985; Kelman et al. 1998). We, too, recorded zones of any size as positive results, since the diameter of the zone is partially related to the ability of metabolites to diffuse into the agar, and lipid-soluble compounds will often diffuse into the agar less well than more polar compounds (Walls et al. 1993). However, there is no doubt that small zones of inhibition need to be interpreted with caution (Jenkins et al. 1998). Physical and chemical characteristics of the extract such as viscosity or pH can inhibit growth of bacteria and also primary metabolites can exhibit weak inhibitory effects when tested at high concentrations (Jensen et al. 1996; Jenkins et al. 1998). In our study, a few extracts (*Flabellina salmonacea*, *Halocynthia pyriformis*, *Styela* spp.) caused exclusively very small inhibition zones, and while the antimicrobial activity of *H. pyriformis* was confirmed through experiments with increased extract concentrations, further experiments with extracts from *Styela* spp. and *F. salmonacea* are still lacking. If small inhibition zones were to be disregarded, then only 22% of the investigated species show moderate or high inhibition of at least one bacterial strain at natural extract concentrations.

The crude extract of the sponge *Haliclona viscosa* exhibited considerably stronger inhibition of bacteria and affected more bacterial strains than any other extract tested. The strong activity against a variety of different bacterial strains suggests broad-spectrum active compounds although Newbold et al. (1999) mention for Caribbean sponges that broad-spectrum antibacterial agents are uncommon. Bioassay-guided fractionation of the crude extract revealed that the strong activity in *H. viscosa* was caused by compounds of the *n*-BuOH/EtOAc fraction. However, none of the purified compounds (X2, X3, X4) alone repeated the effect of the *n*-BuOH/EtOAc fraction. The effect of X2, a compound that also deterred feeding of sympatric amphipods (Lippert et al. in prep.) was most comparable to the effects of the crude extract. Surprisingly, the only strain not or only weakly inhibited by the three pure compounds was strain C, which was one of the two more sensitive strains in all other experiments. The various combinations of compounds had similarly strong effects as the pure compounds (Table 4) but no additive or synergistic effects were observed to cause the strong effect of the

crude extract. Also Newbold et al. (1999) found inhibition zones produced by crude extracts to be greater than those of purified compounds, and suggests that the crude extract may contain additional minor compounds that were not isolated. Furthermore, extraction procedures may lead to a certain loss of compound mass. Several species of the genus *Haliclona* from tropical and Antarctic regions are reported to contain biologically active compounds (e.g. McClintock, 1987; Baker et al. 1988; Fusetani et al. 1989; Bakus et al. 1994; Jaspars et al. 1994; Charan et al. 1996; Clark et al. 1998; Harrison 1999; Brown et al. 2001). While extracts of *Haliclona cinerea* from California and of *Haliclona mediterranea* from the Mediterranean were highly active against bacteria (Thompson et al. 1985; Amade et al. 1987), other species of this genus have been shown to be inactive or only weakly active against microorganisms (*Haliclona heterofibrosa*, Bergquist and Bedford 1978; *Haliclona* sp., McClintock and Gauthier 1992). From the two species of the genus *Haliclona* (*H. viscosa*, *H. rosea*) occurring in the sub-Arctic Kongsfjord we found only the extract of *H. viscosa* to possess antibacterial activity. A similar difference in activity was found for antifeeding activity against amphipods in extracts of these two species (Lippert et al. in prep.).

In the context of evolutionary questions about bioactive natural compounds and the adaptive influence of environmental and biological factors versus genetically fixed traits, it is an interesting observation that the two closely related species of *Haliclona* in Kongsfjord show very different biological activity. This could suggest that environmental conditions are less important than species-specific traits. In contrast, highly similar activity was found in taxonomically closely related octocorals from opposite polar regions, indicating that environmental factors may be very important. The extract of *Gersemia antarctica*, a close relative to *Gersemia rubiformis* tested in the present study, showed weak (<2 mm) growth inhibition of sympatric bacteria at tissue level concentration and inhibition was, similar to our results, increased at higher tissue level concentration (4-6 mm) (Slattery et al. 1995). Both, *G. antarctica* and *G. rubiformis*, contain bioactive compounds which also deter feeding of sympatric predators (Slattery and McClintock 1995; Lippert et al. in prep.). Biological activity of extracts can also differ within populations of the same species from different locations. The sponge *Suberites ficus* from Kongsfjord did not show antimicrobial activity, but extracts of the same species from southern Britain were active against a marine and a non-marine bacterial strain (Dyrynda

1985). Direct comparisons between these different studies, however, have to be considered cautiously because of the variety of methods used and the different bacterial strains employed. This variety within antimicrobial activity across species and geographic regions shows that evolutionary considerations within chemical ecology will need to be much more rigorously tested before it may be possible to unravel any patterns.

OBSERVATIONS IN NATURE

Deterring microbial attachment and inhibiting microbial growth may be strategies to limit the establishment of later successional fouling stages through the prevention of surface conditioning by early successional bacteria (Thompson et al. 1985; Davis et al. 1989; Wahl 1989; Wahl et al. 1994). From our own underwater observations we saw that in nature the investigated invertebrates were fouled to different degrees with epizoids, although we could not collect quantitative data. In some cases, a relationship between the degree of fouling and the antimicrobial activity seem to exist. For example, the soft coral *Gersemia rubiformis* and the ascidian *Halocynthia pyriformis* were never fouled and also exhibited antibacterial activity. The ascidian *Styela* spp. and the bryozoans *Tricellaria ternata* and *Eucratea loricata* were heavily fouled and did not inhibit bacterial growth. On the other hand, there are several, non-fouled species which also did not show any antibacterial activity in our experiments, like the actinians *Hormatia nodosa*, *Urticina asiatica* and *Urticina eques*, and the sponge *Suberites ficus*. There may be mechanisms other than secondary metabolites responsible for antifouling properties in *Hormathia nodosa* and *Urticina eques*, for example mechanical or physical defenses like tissue sloughing (Barthel and Wolfrath 1989) or mucus secretion (Krupp 1985).

Similar observations were made if we compare the number of bacterial strains that could be cultured from swabbing similarly sized surface areas of invertebrates. This is by no means a quantitative measure of the actual degree of microbial fouling and it only refers to those bacterial strains that grow under culture conditions. We found, however, that the actinians *Hormathia nodosa* and *Urticina eques* yielded very few bacteria in culture while some other species, e.g., the

ascidian *Stylea* spp., that were overgrown with fouling macroorganisms also yielded a large number of cultivable surface bacteria.

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