Screening for antibacterial and antifungal activities in marine benthic invertebrates from northern Norway

Margey Tadesse[a], Bjørn Gulliksen[b], Morten B. Strøm[c], Olaf B. Styrvold[a] and Tor Haug[a]

[a]*Department of Marine Biotechnology and Centre for Research-based Innovation on Marine Bioactivities and Drug Discovery (MabCent), The Norwegian College of Fishery Science, University of Tromsø, Breivika, N-9037 Tromsø, Norway*

[b]*Department of Aquatic Biosciences and MabCent, The Norwegian College of Fishery Science, University of Tromsø, Breivika, N-9037 Tromsø, Norway*

[c]*Department of Pharmacy and MabCent, Faculty of Medicine, University of Tromsø, Breivika, N-9037 Tromsø, Norway*

Corresponding author: Tel: +47 77 64 67 28; Fax: +47 77 64 51 10

Email address: margey.tadesse@nfh.uit.no (M. Tadesse)

Abstract

Benthic marine invertebrates collected from sub-Arctic regions of northern Norway, were found to be a promising source of novel bioactive compounds against human and fish pathogenic bacteria and fungi. Lyophilized material from seven species of ascidians, six sponges and one soft alcyonid coral were extracted with 60% acidified acetonitrile (ACN). After separation into an ACN-rich phase (ACN extract) and an aqueous phase, and subsequent solid phase extraction of the aqueous phase, fractions differing in polarity were obtained and screened for antibacterial and antifungal activities, along with the more lipophilic ACN-extracts. Antimicrobial activity was determined against two Gram-negative, two Gram-positive bacteria, and two strains of fungi. Notably, all the invertebrate species in

the study showed activity against all four strains of bacteria and the two strains of fungi. In general, the aqueous fractions displayed highest antimicrobial activity, and the most potent extracts were obtained from the colonial ascidian *Synoicum pulmonaria* which displayed activity against bacteria and fungi at a concentration of 0.02 mg/ml; the lowest concentration tested.

Keywords: Antimicrobial screening; Marine bioprospecting; Natural products; Sponges; *Synoicum pulmonaria*; Tunicates

1. Introduction

The evolution of antibiotic-resistant bacteria has stimulated the search for potent antibacterial agents from natural sources. While natural products have traditionally been harvested from terrestrial sources, reports show that more than 15.000 marine natural products have been isolated in the period from 1965 to 2005 (Blunt et al., 2007). A major contributing factor to this development is the fact that modern technology has made it easier to gain access to the great biodiversity of life found in the oceans (Battershill et al., 2005).

Since the early days of marine natural product discovery, *Porifera* (sponges) and *Chordata* (including ascidians) have dominated as the major contributing phyla of novel bioactive compounds (Blunt et al., 2007). Sponges, in particular, are responsible for a large number of these compounds, which exhibit a wide range of activities including antitumor (Baslow and Turlapaty, 1969), antiviral (Carter and Rinehart, 1978), antibacterial (Sharma and Burkholder, 1967) and antifungal activity (Phillipson and Rinehart, 1983). The compounds also show broad chemical diversity and are among others comprised of unusual nucleosides (Quinn et al., 1980), terpenes (Cimino et al., 1971), peptides (Stonard and Andersen, 1980), alkaloids (Braekman et al., 1982), fatty acids (Morales and Litchfield, 1976), and unnatural amino acid (which are frequently halogenated) (Crews et al., 1986; Inman and Crews, 1989). It is believed that the early appearance of sponges in evolution has afforded them sufficient time to develop an advanced chemical defence system (Sipkema et al., 2005). A number of bioactive compounds have also been isolated from ascidians, exhibiting activities such as antiviral (Rinehart et al., 1984), cytotoxic (Moquin-Pattey and Guyot, 1989) antibacterial (Azumi et al., 1990) and enzyme inhibitory activities (Sato et al., 1998). These compounds are mainly comprised of various derivatives of alkaloids and peptides. There are a few examples of marine derived compounds which have successfully reached the market as

therapeutic drugs. Two nucleoside analogues (originally isolated from the Caribbean sponge *Cryptotethya cryta* in the 1950s) are today in clinical use; the antiviral compound Ara-A and the anticancer compound Ara-C (cytarabine) (Newman et al., 2000). Trabectedin (Yondelis; ET-743), a synthetic antineoplastic agent derived from the Caribbean tunicate *Ecteinascidia turbinata*, is currently used in Europe for treatment of advanced soft tissue sarcoma and ovarian cancer (Carter and Keam, 2007). Reports of antitumor activity of extracts of *E. turbinata* date back to 1969, however, characterization of E-743 and related compounds was not achieved until the end of the 1980s (Rinehart et al, 1990). Ziconotide (Prialt) is the synthetic equivalent of a peptide first purified in 1984 from the venom of a marine mollusk *Conus geographus* (Olivera et al, 1984). The drug was approved in the United States in 2004 and in the European Union in 2005 for the management of severe chronic pain (Skov et al, 2007).

The primary focus for marine drug discovery has been the tropical and temperate Atlantic and Pacific regions. Polar regions, such as the North-Atlantic oceans, the Barents Sea and the Arctic Sea are relatively unexplored marine environments. In addition, Norway possesses an abundance of biota along its long coastline that has barely been investigated for bioactive compounds. The present work has its origin off the coast of northern Norway, in which seven species of ascidians, six species of sponges and one soft alcyonid coral were screened for antibacterial and antifungal activities. The project is a first step towards screening and later structural elucidation of novel marine compounds from this cold-water region with activity against human and fish pathogenic bacteria and fungi. Resistant strains of the Gram-negative bacteria *Escherichia coli* and the Gram-positive *Staphylococcus aureus*, along with coagulase-negative staphylococci are responsible for over 50% of hospital bloodstream infections (Bax et al., 2000). Furthermore, the Gram-negative bacteria *Listonella (Vibrio) anguillarum* is a fish pathogenic bacterium which causes substantial financial loss to

the aquaculture industry (Toranzo et al., 2005). Clinical infection by the fungus *Candida albicans* due to transplantation procedures, immunosuppression, the use of chronic medical indwelling devices, prolonged intensive care unit stays, and systemic disease, has a mortality rate as high as 40% (Chandra et al., 2001). *Saccharomyces cerevisiae* has been implicated in fatal systemic disease in immunocompromised patients such as HIV and cancer patients (Murphy and Kavanagh, 1999). Thus, there is an urgent need for novel chemical entities against both Gram-negative and Gram-positive bacteria and fungi. Also included in the screening assay was the Gram-positive bacterium *Corynebacterium glutamicum* which is recognized as a particularly sensitive test-bacterium for the detection of antibacterial activity in samples with low concentrations of active components (Haug et al., 2002).

The results of the present study showed that highly potent antibacterial and antifungal activities could be detected in extracts from all species investigated. The fractions and extracts prepared were complex and in most cases probably contained a multitude of compounds. Nevertheless they showed a surprisingly broad spectrum of antibacterial and antifungal activities. The most potent fractions displayed antibacterial and antifungal activity at concentrations as low as 0.02 mg/ml. Extracts from the ascidian *Synoicum pulmonaria* were especially efficacious, showing full inhibition of microbial and fungal growth at the lowest concentration tested, making this species a promising candidate for further studies.

2. Materials and methods

2.1. Experimental animals and sample collection

Live specimens of sub-Arctic marine benthic invertebrates were obtained off the coast of northern Norway in the period from November 2005 until April 2006. All 14 species are relatively common in coastal waters of northern Norway. Samples of each species were identified, pooled, lyophilized and separately frozen at -20 ^{o}C. Associated macroorganisms (mainly algae, polychaetes and ophiuroids) were removed from the biological material before lyophilisation.

The benthic organisms analyzed were seven species of ascidians, six species of sponges and one soft coral. The ascidian material consisted of six solitary (*Ascidia virginea*, *Ciona intestinalis*, *Corella parallelogramma*, *Dendrodoa aggregata*, *Halocynthia pyriformis* and *Styela rustica*) and one colonial (*Synoicum pulmonaria*) species. All ascidian species are epifaunal, usually found attached to bedrock or stones. Three of the six species of sponges could be identified to species level (*Geodia barretti, Haliclona rosea* and *Myxilla incrustans*). The first two species are commonly found attached to bedrock while *M. incrustans* was collected as epigrowth of the bivalve *Chlamys islandica*. The three remaining sponges belong to two genera, namely *Haliclona* (2 spp.) and *Polymastia*. The colonial soft alcyonid coral (*Alcyonium digitatum*) also prefers bedrock as habitat and was collected in a tidal rapid.

2.2. Extraction

Lyophilized samples (0.79-25 g) were extracted with 10 volumes (v/w) of 60% acetonitrile (ACN; HPLC-grade, VWR International, Leuven, Belgium) in Milli-Q water (v/v)

containing 0.1% triflouracetic acid (TFA; Merck, Damstadt, Germany) for 24 h at 4 ^{o}C. The supernatant was collected, stored at 4 ^{o}C, and the residue extracted once more under the same conditions. The combined supernatants were incubated at -20 ^{o}C for 1-2 h allowing separation into two liquid phases, an ACN-rich phase (hereafter called ACN-extracts) and an aqueous (salt-rich) phase. This separation is caused be the high salt content in the invertebrate tissues, which is immiscible with ACN. After separation of the two phases and lyophilisation, the ACN-extract was stored at –20 ^{o}C until activity screening was performed, while the remaining pellet of insoluble material was discarded. The aqueous phase was dissolved in Milli-Q water to a concentration of 100 mg/ml. To avoid false positives during antimicrobial testing, salts were removed from the aqueous phase by solid-phase extraction (SPE), as described by Haug et al. (2002). Briefly, the aqueous phase was loaded onto Sep-Pak C18 Vac cartridges (Waters) equilibrated in acidified Milli-Q water (0.05% TFA). After washing with acidified water, three stepwise elutions were performed with acidified solutions of 10%, 40%, and 80% ACN in Milli-Q water (v/v) (containing 0.05% TFA). Non-bound material was discarded. The isolated SPE-fractions from the aqueous phase were lyophilized and kept frozen at -20 ^{o}C until bioactivity screening was performed. Figure 1 gives a summary of the extraction and purification procedures employed in the study.

2.3. Antibacterial assay

The Gram-negative bacteria *Listonella anguillarum*, serotype O2 (FT 1801, a fish pathogenic strain), *Escherichia coli* (ATCC 25922), and the Gram-positive bacteria *Staphylococcus aureus* (ATCC 9144) and *Corynebacterium glutamicum* (ATCC 13032) were used as test organisms. All isolates were grown at room temperature (18-20 ^{o}C) in Mueller Hinton Broth (MHB; Difco Laboratories, Detroit, USA). The SPE-fractions and the ACN-

extracts were diluted in Milli-Q water to a concentration of 10 mg/ml and serial two-fold dilutions were performed prior to testing for antibacterial activity. The antibacterial activities were determined by continuous monitoring of bacterial growth with a Bioscreen C microbiology reader (Labsystems Oy, Helsinki, Finland), as described by Haug et al. (2004). The test was performed in 100-well flat-bottomed honeycomb plates, in which 50 μl of test fractions were incubated with 50 μl of a suspension of an actively growing (log phase) culture of bacteria diluted to a starting concentration of approximately 5×10^3 cells per well. The growth chamber was maintained at 20 ^{o}C during the incubation period. The absorbance was measured at 2 h intervals for 72 h by a turbidimetric method with vertical light photometry and a wide band filter (420–580 nm), which is less sensitive to colour change in the sample compared to single wavelength detection. Cecropin B (25 μM), an antimicrobial peptide originally isolated from the silk moth *Hyalophora cecropia*, was synthesized as described by Kjuul et al. (1999) and used as a positive control. Antibacterial activity was determined when the optical density of the growth control (bacteria plus water) reached an OD of approximately 0.3. Fractions were regarded as active when the optical density was less than 50% of the growth control.

2.4. Antifungal assay

Saccharomyces cerevisiae was a gift from Dr. Arne Tronsmo (The Norwegian University of Life Sciences, Ås, Norway) whereas *Candida albicans* was obtained from the American Type Culture Collection (ATCC 10231). Both fungi were cultivated on potato dextrose agar with 2% glucose at room temperature. Fungal spores were dissolved in potato dextrose broth (Difco) and the cell concentration was determined and adjusted after counting in a Bürker chamber. An aliquot of 50 μl of fungal spores (final concentration 2 x 10^5

spores/ml) were inoculated in 96-well nunc™ microtitre plates along with 50 µl of the SPE-fractions or ACN-extracts which were dissolved in Milli-Q water. The SPE-fractions and ACN-extracts were tested at final concentrations (prepared from serial two-fold dilutions) ranging from 5 to 0.02 mg/ml. Cultures were grown in a moist dark chamber without shaking at 20 °C for *S. cerevisiae* and at 37 °C for *C. albicans*. Synthetic cecropin B was used as a positive control (3.12 µM against *S. cerevisiae* and 6.25 µM against *C. albicans*). Growth inhibition was determined microscopically after 48 h of incubation. MIC (minimal inhibitory concentration) was set as the lowest concentration of sample resulting in more than 50% inhibition of visible growth compared to the growth control (fungal spores plus water). Fractions that were active at concentrations of 0.08 mg/ml were considered to possess high activity.

3. Results

Tables 1 and 2 show the results from the *in vitro* antibacterial and antifungal screening of 56 fractions/extracts from seven ascidians, six sponges and one soft alcyonid coral collected off the coast of northern Norway. All species tested yielded fractions showing activity against both bacteria and fungi. Overall, the aqueous fractions (especially the 40% and 80% SPE-fractions) displayed highest antimicrobial activity. Furthermore, the Gram-positive bacterial test strains were in general the most sensitive microorganisms (Table 3).

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3.1. Antibacterial and antifungal activity in ascidians

Seven species of ascidians were investigated for antibacterial activity (Table 1). A total of 23 fractions (including SPE-fractions and ACN-extracts), including SPE-fractions and ACN-extracts, showed antibacterial activity against both of the Gram-negative bacteria (*E. coli* and *L. anguillarum*). Within these, five of the fractions were active at a concentration of 0.08 mg/ml (Table 3). Notably, *L. anguillarum* was more susceptible than *E. coli*, and except for the 10% SPE-fraction of *C. intestinalis*, all SPE-fractions displayed antibacterial activity against *L. anguillarum*. Among the ascidians, the highest activity against the Gram-negative bacteria were obtained in the 40% and 80% SPE-fractions from *C. intestinalis* and the 80% SPE-fraction from *S. pulmonaria* and *D. aggregata*. Notably, most of the more lipophilic ACN-extracts from the ascidians showed no antibacterial activity against the Gram-negative bacteria within the concentration range tested. The only exceptions being the ACN-extracts from *S. pulmonaria, D. aggregata* and *A. virignea*, in which the extract from *S. pulmonaria* was the overall most potent ACN-extract obtained against the Gram-negative bacteria with an antibacterial activity at 0.02 mg/ml.

Overall, the SPE-fractions from the ascidians exhibited a higher potency against the Gram-positive bacteria than against the Gram-negative bacteria, and as expected *C. glutamicum* was more susceptible than *S. aureus* (Table 1). A total of 25 fractions displayed activity against the Gram-positive bacteria, and 11 fractions displayed high activity (Table 3). The 40% and 80% SPE-fractions of *S. pulmonaria*, *C. intestinalis*, *D. aggregata* and *S. rustica,* as well as the 10% SPE-fractions of *D. aggregata* and the 40% SPE-fraction of *A. virginea*, all gave high activity against the Gram-positive bacteria. Except for the ACN-extracts of *S. rustica* and *H. pyriformis*, all the more lipophilic ACN-extracts showed antibacterial activity.

As observed against the Gram-negative bacteria, the most potent SPE-fractions and ACN- extracts were obtained from the colonial ascidian *S. pulmonaria*. Notably, the ACN-extract inhibited growth of all the Gram-negative and Gram-positive bacteria at a concentration of 0.02 mg/ml (Table 1). Fig. 2 shows the time course study of the antibacterial effect of the ACN-extract at the three lowest concentrations tested, and illustrates the high efficiency of this extract, as no bacterial growth is detected.

In addition to antibacterial activity, we also screened the SPE-fractions and ACN-extracts for antifungal activity. Notably, we detected *in vitro* antifungal activity in all ascidian species tested, and *S. cerevisiae* was in general more susceptible than *C. albicans* (Table 1). The SPE-fractions and the ACN-extracts showing high activity against bacteria also exhibited high activity against fungi, although the overall potency against fungi was lower than against bacteria. The ACN-extract from *S. pulmonaria* showed high activity against *C. albicans* and *S. cerevisiae* (Fig. 3). Similarly to the antibacterial results, the 40% and 80% SPE-fractions possessed the highest potencies. The 40% and 80% SPE-fractions of *C. parallelogramma* were the only fractions that exhibited higher activity against fungi than bacteria.

3.2. Antibacterial and antifungal activity in sponges

Six marine sponges were screened for antibacterial activity (Table 2). A total of 19 fractions from the sponges had an activity against the Gram-negative bacteria within the range tested, while two fractions were highly active (Table 3). As observed for the ascidians, the sponge fractions/extracts displayed higher potency against *L. anguillarum* than against *E. coli*. The most potent SPE-fractions against the Gram-negative bacteria were the 40% SPE-fractions from the sponges *G. barretti* and *Haliclona* sp. 2. The 40% and 80% SPE-fractions of the *Polymastia* sp. and the 80% SPE-fraction of *M. incrustans* exhibited an activity against *L. anguillarum* at a concentration of 0.63 mg/ml. The ACN-extracts were in general found to be less potent than the 40% and 80% SPE-fractions.

Similarly to the ascidians, the sponge species tested tend to exhibit higher antibacterial activity against the Gram-positive bacteria than against the Gram-negative bacteria. A total of 21 fractions obtained from the sponge species tested showed activity against the Gram-positive bacteria, and among these three fractions were highly active (Tables 2 and 3). The 40% SPE-fraction of *G. barretti* showed exceptionally high potency against both *C. glutamicum* and *S. aureus* at a concentration of 0.04 mg/ml. The 40% SPE-fraction of *Haliclona* sp. 2 inhibited the growth of both Gram-positive strains at a concentration of 0.08 mg/ml.

In vitro antifungal activity was found in all species of sponges tested. As for the ascidians, the sponges generally exhibited higher activity against *S. cerevisiae* than against *C. albicans*. A total of eighteen sponge fractions showed activity against fungi, and 2 fractions showed high activity (Table 3). As in the case of the ascidians, the sponge species also displayed higher activity against bacteria than fungi. The exceptions were the 40% and 80% SPE-fractions of *H. rosea*, which exhibited significantly higher activity against *S. cerevisiae*

than against most of the bacterial strains. Several fractions of *Geodia barretti*, *Haliclona* sp. 2, *M. incrustans* and *Polymastia* sp. inhibited fungal growth at a concentration 0.63 mg/ml or less.

3.3. Antibacterial and antifungal activity in the soft acyonid coral Alcyonium digitatum

As observed for the ascidians and the sponges, the soft alcyonid coral showed higher activity against Gram-positive bacteria than Gram-negative bacteria, where it exhibited activity at a concentration of 0.08 mg/ml (Table 2). Antifungal activity was observed in all the SPE-fractions of *A. digitatum*.

4.0 Discussion

The colonial ascidian species *S. pulmonaria* gave the highest activities detected against both bacteria and fungi, and at a concentration of 0.02 mg/ml. This was the lowest concentration tested in the study, indicating that the potency was possibly higher. It is interesting that the ACN-extract of *S. pulmonaria* gave strong activity when there was generally low activity in the ACN-extracts of the other organisms tested, suggesting that more lipophilic components might be responsible for the activity observed. Antibacterial and antifungal activities have to our knowledge not been reported in *S. pulmonaria* previously.

The 40% and 80% SPE-fractions of the ascidian *C. intestinalis*, had high activities against both bacteria and fungi. It is noteworthy that two antimicrobial peptides, an 8-15 kDa peptide active against marine Gram-positive bacteria, and a 60-70 kDa protein, active against both marine Gram-positive and Gram-negative bacteria, have been isolated from the blood cells of *C. intestinalis* (Findlay and Smith, 1995). Fedders and Liepe (2008) recently confirmed these findings, however, they were unable to identify the peptides structurally by *N*-terminal protein sequencing and mass spectrometric methods due to an unfortunate combination of post-translational modifications, subspecies differences and low abundance (Fedders and Leippe, 2008). However, using genomic data they identified a putative gene family exhibiting features typical of antimicrobial peptides and synthesized a peptide based on these data which showed activity against both Gram-negative and Gram-positive bacteria (Fedders and Leippe, 2008). An additional synthetic peptide, M4F19, showing antimicrobial activity, has been prepared based on a putative antimicrobial precursor peptide, identified in the blood cells of *C. intestinalis* (Jang et al., 2005). Whether the antibacterial activity detected in the present study is caused by antimicrobial peptides or other compounds remains to be clarified. Antifungal activity has been reported for *C. intestinalis* in the form of

tribromophenol, which is a known fungicide (Kotterman et al., 2003). In the former study, *C. intestinalis* was collected from a recirculation system in the Netherlands and extracted with a relatively lipophilic mixture of hexane/acetone (3:1) (Kotterman et al., 2003). The antifungal activity detected in the aqueous SPE-fractions in the present study might therefore be caused by other, more hydrophilic compounds.

Antibacterial activity has previously been detected in methanol/dichloromethane extracts of the ascidians *H. pyriformis* and a mixture of two *Styela* species where one of the species was *S. rustica* (Lippert et al., 2003). The species in the study by Lippert et al. (2003) were collected in Spitsbergen, Norway, which is located about 10^{o} north of where the species in our project were collected. Interestingly, they screened 18 invertebrate species (including ascidians, sponges and bryozoa), but only 7 species showed antibacterial activity. Lippert et al (2003) suggested that only a minor portion of Arctic invertebrates possess antibacterial activity compared to species from lower latitudes, as well as those from Antarctica. This statement is strongly contradicted by the findings in the present study where all species screened displayed antibacterial activity. However, the difference in the incidence of activity may be due to differences in methods of extraction, bacterial strains tested, etc. To our knowledge, antibacterial and antifungal activities have previously not been reported for the other cold-water ascidians investigated in the current project.

The sponge *G. barretti*, collected from the coast of Sweden, has previously been shown to possess activity against *E. coli* and *S. aureus* (Andersson et al., 1983). The activity was detected in a water and a petroleum ether extract. Two antifouling cyclopeptides, barettin and 8,9-dihydrobarettin, were later isolated from *G. barretti* by the same research group (Sjogren et al., 2004). These compounds might be responsible for the antimicrobial activities detected in the present study.

There are some reports of antibacterial activity in the sponge genera *Haliclona* collected from cold water regions. These include extracts of the sponge *H. viscosa* collected from Spitsbergen, Norway, in the study by Lippert et al (2003). In later work, Volk and Kock (2004) structurally characterized an antimicrobial compound isolated from an *n*-butanol extract of the same species, collected from the same area of Norway. An aqueous extract of *H. rosea* (from the Mediterranean) was shown to be active against *E. coli*, whereas methanol and acetone extracts had no activity (Nigrelli et al., 1967). Lippert et al. (2003) also investigated antimicrobial activity in *H. rosea*, but detected no antimicrobial activity. However, this may be due to different strains of test-bacteria and/or different experimental procedures. Antifungal compounds have also been previously isolated from *Haliclona* species. An alkaloid isolated from *Haliclona* was found to be active against *C. albicans* (Fahy et al., 1988). A sphingosine derivative, also derived from a *Haliclona* species, exerted activity against *S. cerevisiae* (Richelle-Maurer et al., 2001).

Antimicrobial activities have also been shown in several sponges of the genera *Polymastia*, sampled from Polar Regions. *P. invaginata* collected from Antarctica, has been shown to inhibit both Gram-negative and Gram-positive bacteria (McClintock and Gauthier, 1992). A steroid/amino acid conjugate, Polymastiamide A, isolated from the Norwegian sponge, *P. boletiformis*, was found to be active against various human pathogens including *S. aureus* and *C. albicans* (Kong and Anderssen, 1993).

Ethanol extracts of the sponge *M. incrustans*, collected off the coast of Mexico, displayed no antibacterial and antifungal activity (Encarnacion et al., 2000). This is in contradiction to the findings in the current work where *M. incrustans* displayed activity against both bacteria and fungi. It is worth pointing out that we can not exclude symbiotic microorganisms as the source of the active compounds instead of the invertebrate species

themselves. For instance, a fungal strain isolated from *M. incrustans* has been shown to produce numerous antimicrobial compounds (Holler et al, 1999).

In the present study, the soft alcyonid coral *A. digitatum* displayed potent antimicrobial activity against both bacteria and fungi. Activity against *S. aureus* has previously been reported in the water extract of *A. digitatum* (Andersson et al., 1983). Antibacterial activity was also detected in the related species *A. paessleri* (Slattery et al., 1995).

Sponges and ascidians have been subjects of several antimicrobial screening studies. Rinehart *et al*. (1981) examined 71 unidentified species of sponges from the west coast of Baja California and the Gulf of California and found 52% to have antimicrobial activity. In the same study, 82% out of 187 Caribbean sponges showed antimicrobial (including antiviral) activity. Munro *et al*. (1989) examined 302 species of sponges collected off the coast of New Zealand, Antarctica and Western Samoa and found 28% to have antimicrobial activity. In the same study, 41% out of 80 ascidians displayed antimicrobial activity. The concentration ranges for the extracts tested in the screening studies are not stated. The findings in the current work appear to be unique compared to the bulk of the literature in that activity is found in all of the species screened. Between 1986 and 2005, the NCI Developmental Therapeutics Program (DTP) found antileukemic activity against six leukemic cell lines at a concentration of 0.1 mg/ml in 9% of the marine sponge species tested (Cragg et al., 2006). In the current work, antibacterial activity at a concentration of 0.08 mg/ml (defined as high activity), was found in 50% of the sponge species tested and 71% of the ascidian species. Although different species and experimental procedures have been used in the different studies, the data presented provide an indication of the high frequency of detectable antimicrobial activity in marine sponges and ascidians. Altogether, these results suggest that marine sponges and ascidians are a valuable source for the discovery of new types of antibiotics.

Many antimicrobial screening studies have shown that Gram-positive bacteria are more sensitive than Gram-negative bacteria and fungi to extracts of sponges and ascidians (Amade et al., 1987; McCaffrey and Endean, 1985; Rinehart et al., 1984). In fact, out of 777 species of sponges collected from the Caribbean Sea, 35% had activity against Gram-positive bacteria, 15% had activity against Gram-negative bacteria, and 10% had activity against *Candida* sp. (Burkholder, 1968). This is in agreement with our results, where 29% of the total number of fractions/extracts tested showed strong activity (active at a concentration of 0.08 mg/ml) against Gram-positive bacteria, whereas 13% of the fractions/extracts tested were active against Gram-negative bacteria and 5% were active against fungi at the same concentration (Table 3).

The fact that antibacterial and antifungal activities were detected in all species tested, points to the high efficiency of the extraction method which is relatively straightforward and rapid. The high incidence of activity detected by this method compared to most of the extraction methods found in the literature could be due to the relative polar nature of the extraction solvent (60% ACN). The modest activity observed in the ACN-rich extracts supports this theory. The effect of different solvents on antibacterial activity measurements is well illustrated in the study by Constantine et al. (1975). They extracted 25 different invertebrates successively with petroleum ether, followed by ethanol and ethanol-water (1:1) and investigated the antibacterial activity in each extract. No activity was detected in the petroleum ether extracts, whereas 56% of the ethanol extracts and a 100% of the ethanol-water extracts, displayed antibacterial activity. Fusetani (1988) extracted 282 sponge species with methanol and partitioned the evaporated extract into a lipophilic and aqueous fraction using chloroform and water. Overall, 21% of the species displayed antifungal activity, with the aqueous extracts showing highest activity. These results are in agreement with our results, showing highest activity in the polar, aqueous fractions. It is also striking that the slightly

more lipophilic 40% and 80% SPE-fractions, were generally more potent against both bacteria and fungi than the polar 10% SPE-fractions (Table 3).

Preliminary experiments (data not shown) show that most of the fractions, which were all extracted according to the procedure outlined in Fig. 1, contained mixtures of compounds. Given that these are multi-component fractions, there is a potential for discovering multiple active compounds in a single fraction, making these organisms a valuable source of novel substances for future drug development. A synergistic effect arising from several compounds with similar polarity/lipophilicity, can also account for the high activities that are observed. The excellent activities observed in some of the fractions could stem from low-abundant substances, signifying a high potency for these compounds. Work is currently being carried out for the isolation and structural elucidation of the active compounds.

4. Conclusion

This is the first report of a screening of antibacterial and antifungal activities in marine benthic sedentary ascidians and sponges collected off the coast of northern Norway. Seven different ascidian species, six sponge species and one soft alcyonid coral species, were extracted and screened for activity. The results showed that highly potent antibacterial and antifungal activities could be detected in all species investigated. Thus, cold-water regions such as the coast of northern Norway are rich sources of potent antibacterial and antifungal compounds. Among the species investigated, *S. pulmonaria* was the most promising species tested by providing highly active SPE-fractions and ACN-extracts against both Gram-positive and Gram-negative bacteria, as well as fungi. Thus, these fractions are very promising for further efforts on purification and isolation of single bioactive compounds for future drug development.

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Table 1. Antibacterial and antifungal activities in SPE-fractions (SPE) and ACN-extracts (ACN extr) from marine ascidians. Antibacterial activity was tested against *Listonella anguillarum (La)*, *Escherichia coli* (*Ec*), *Corynebacterium glutamicum* (*Cg*) and *Staphylococcus aureus* (*Sa*). Antifungal activity was tested against *Candida albicans* (*Ca*) and *Saccharmoyces cerevisiae* (*Sc*). Minimal inhibitory concentration (MIC) was defined as the concentration resulting in 50% inhibition of visible growth compared to a growth control.

Species	Fraction[1]	Antimicrobial activity (MIC; mg/ml)				Antifungal activity (MIC; mg/ml)	
		La	*Ec*	*Cg*	*Sa*	*Ca*	*Sc*
Synoicum pulmonaria	10% SPE	1.25	5	1.25	0.16	0.63	1.25
	40% SPE	0.63	0.16	0.16	0.08	0.31	0.63
	80% SPE	0.08	0.08	0.04	0.02	0.31	0.16
	ACN extr	0.02	0.02	0.02	0.02	0.04	0.02
Ciona intestinalis	10% SPE	-	-	-	-	-	-
	40% SPE	0.08	0.08	0.08	5	0.63	0.63
	80% SPE	0.08	0.08	0.08	5	0.16	0.16
	ACN extr	-	-	0.63	-	-	-
Dendrodoa aggregata	10% SPE	0.31	0.31	0.16	0.08	-	5
	40% SPE	5	5	0.08	0.31	0.63	0.31
	80% SPE	0.08	0.31	0.08	0.08	2.5	1.25
	ACN extr	0.31	0.31	0.16	0.16	-	5
Styela rustica	10% SPE	2.5	5	1.25	0.16	1.25	2.5
	40% SPE	1.25	-	0.31	0.08	-	2.5
	80% SPE	1.25	-	1.25	0.08	-	5
	ACN extr	-	-	-	-	-	-
Ascidia virginea	10% SPE	1.25	5	1.25	1.25	0.63	0.63
	40% SPE	0.16	2.5	0.16	0.08	2.5	1.25
	80% SPE	0.31	5	1.25	0.31	1.25	0.16
	ACN extr	0.31	-	2.5	1.25	-	-
Corella parallelogramma	10% SPE	0.63	-	0.63	1.25	-	-
	40% SPE	0.63	2.5	1.25	1.25	0.63	0.16
	80% SPE	5	-	2.5	5	2.5	0.16
	ACN extr	-	-	5	2.5	-	-
Halocynthia pyriformis	10% SPE	5	-	2.5	5	-	-
	40% SPE	5	-	5	-	-	-
	80% SPE	2.5	-	-	2.5	5	5
	ACN extr	-	-	-	-	-	-

Abbreviations: -, no activity. [1]SPE-fractions (SPE) and ACN-extracts (ACN extr).

Table 2. Antibacterial and antifungal activities in SPE-fractions (SPE) and ACN-extracts (ACN extr) from six marine sponges and the soft coral (*Alcyonium digitatum)*. Antibacterial activity was tested against *Listonella anguillarum* (*La*), *Escherichia coli* (*Ec*), *Corynebacterium glutamicum* (*Cg*) and *Staphylococcus aureus* (*Sa*). Antifungal activity was tested against *Candida albicans* (*Ca*) and *Saccharmoyces cerevisiae* (*Sc*). Minimal inhibitory concentration (MIC) was defined as the concentration resulting in 50% inhibition of visible growth compared to a growth control.

Species	Fraction[1]	Antibacterial activity (MIC; mg/ml)				Antifungal activity (MIC; mg/ml)	
		L a	*Ec*	*Cg*	*S a*	*Ca*	*S c*
Geodia barretti	10% SPE	5	5	2.5	5	5	0.63
	40% SPE	0.08	0.31	0.04	0.04	0.63	0.31
	80% SPE	0.31	1.25	0.31	0.16	0.63	0.16
	ACN extr	2.5	-	1.25	1.25	-	-
Haliclona sp. 1	10% SPE	-	-	1.25	-	5	2.5
	40% SPE	-	-	-	-	-	-
	80% SPE	5	-	2.5	0.31	2.5	-
	ACN extr	-	-	-	-	-	-
Haliclona sp. 2	10% SPE	1.25	5	0.31	1.25	1.25	0.31
	40% SPE	0.08	0.16	0.04	0.08	0.31	0.16
	80% SPE	1.25	-	0.31	0.63	2.5	-
	ACN extr	1.25	5	1.25	2.5	2.5	5
Haliclona rosea	10% SPE	1.25	5	1.25	2.5	2.5	0.63
	40% SPE	1.25	5	0.08	5	0.63	0.04
	80% SPE	1.25	5	0.31	0.63	0.63	0.08
	ACN extr	-	-	-	-	-	-
Myxilla incrustans	10% SPE	1.25	2.5	1.25	0.63	1.25	0.63
	40% SPE	1.25	2.5	0.31	1.25	0.63	0.16
	80% SPE	0.63	5	0.63	0.16	1.25	-
	ACN extr	-	-	5	2.5	-	-
Polymastia sp.	10% SPE	2.5	5	1.25	1.25	1.25	0.63
	40% SPE	0.63	2.5	0.16	2.5	0.63	0.31
	80% SPE	0.63	2.5	0.16	0.16	0.31	0.16
	ACN extr	5	-	-	5	-	-
Alcyonium digitatum	10% SPE	1.25	2.5	0.63	0.63	1.25	0.31
	40% SPE	0.63	2.5	0.08	0.31	1.25	0.16
	80% SPE	0.31	-	0.16	0.08	0.63	1.25
	ACN extr	5	-	1.25	5	-	-

Abbreviations: -, no activity. [1] SPE-fractions (SPE) and ACN-extracts (ACN extr).

Table 3. An overview of the antibacterial and antifungal activities found in the SPE-fractions and ACN-extracts. G- and G+ refers to overall antibacterial activity against the Gram-negative and Gram-positive bacteria, while F denotes similar overall antifungal activity.

	Fraction[1]	Number of fractions prepared	Number of fractions showing activity at 5.0 mg/ml			Number of fractions showing activity at 0.08 mg/ml		
			G-	G+	F	G-	G+	F
Ascidians	10% SPE	7	6	6	4	0	1	0
	40% SPE	7	7	7	6	1	5	0
	80% SPE	7	7	7	7	3	4	0
	ACN extr	7	3	5	2	1	1	1
	Total	28	23	25	19	5	11	1
Sponges	10% SPE	6	5	6	6	0	0	0
	40% SPE	6	5	5	5	2	3	1
	80% SPE	6	6	6	6	0	0	1
	ACN extr	6	3	4	1	0	0	0
	Total	24	19	21	18	2	3	2
Coral	10% SPE	1	1	1	1	0	0	0
	40% SPE	1	1	1	1	0	1	0
	80% SPE	1	1	1	1	0	1	0
	ACN extr	1	1	1	0	0	0	0
	Total	4	4	4	3	0	2	0
All	10% SPE	14	12	13	11	0	1	0
	40% SPE	14	13	13	12	3	9	1
	80% SPE	14	14	14	14	3	5	1
	ACN extr	14	7	10	3	1	1	1
	Total	56	46	50	40	7	16	3

[1]SPE-fractions (SPE) and ACN-extracts (ACN extr).

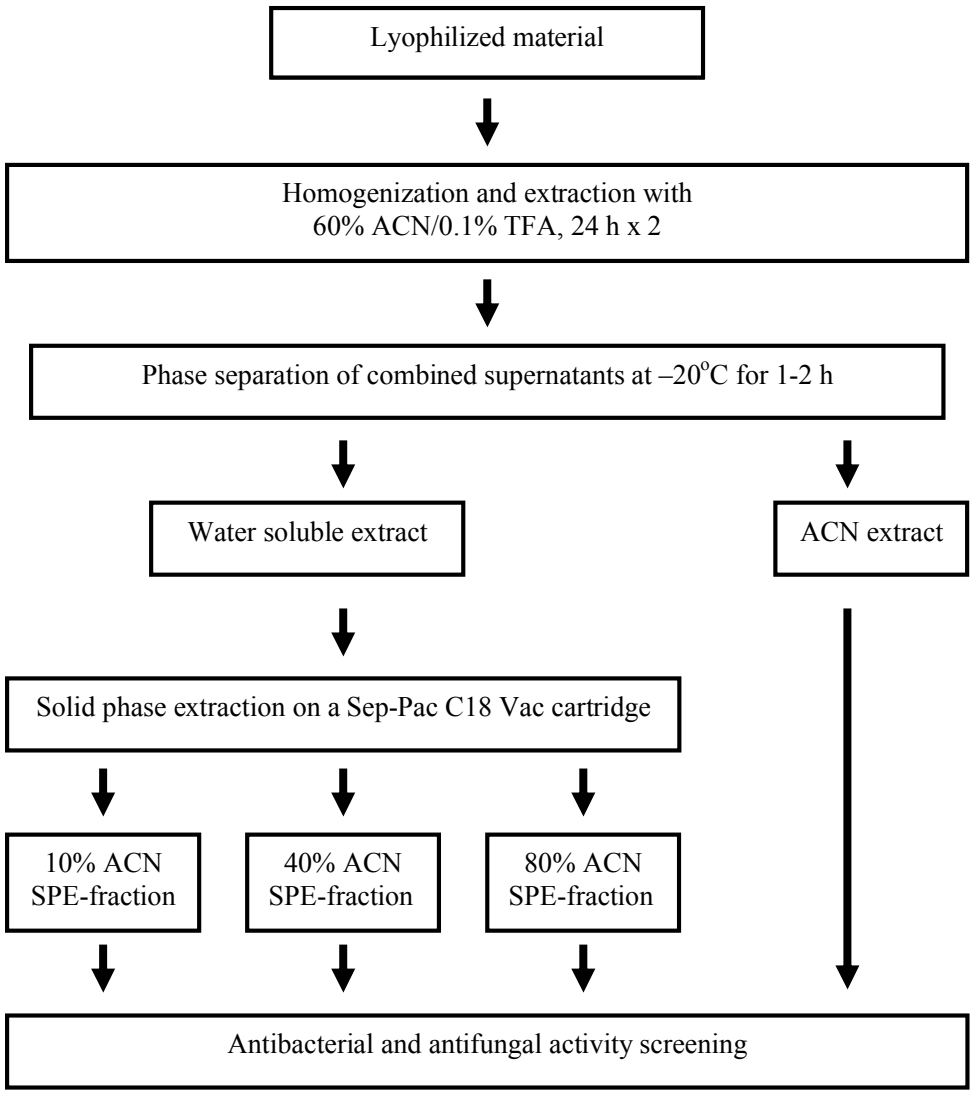

Figure 1. The chart shows a summary of the extraction and purification procedures utilized in the project. Lyophilized material was homogenized and extracted for 24 h with 60% ACN containing 0.1% TFA. The supernatant was removed and the extraction was repeated for another 24 h. The supernatants were combined and incubated at –20 °C for 1-2 h in order for phase separation to occur. The ACN-extract was lyophilised and stored at –20 °C until activity screening was performed, while the water soluble extract was loaded on a Sep-Pac C-18 Vac cartridge to remove salts from the sample. Compounds retained on the column were consecutively eluted with 10%, 40% and 80% ACN, and the resulting SPE-fractions were lyophilised and stored at -20 °C pending activity screening.

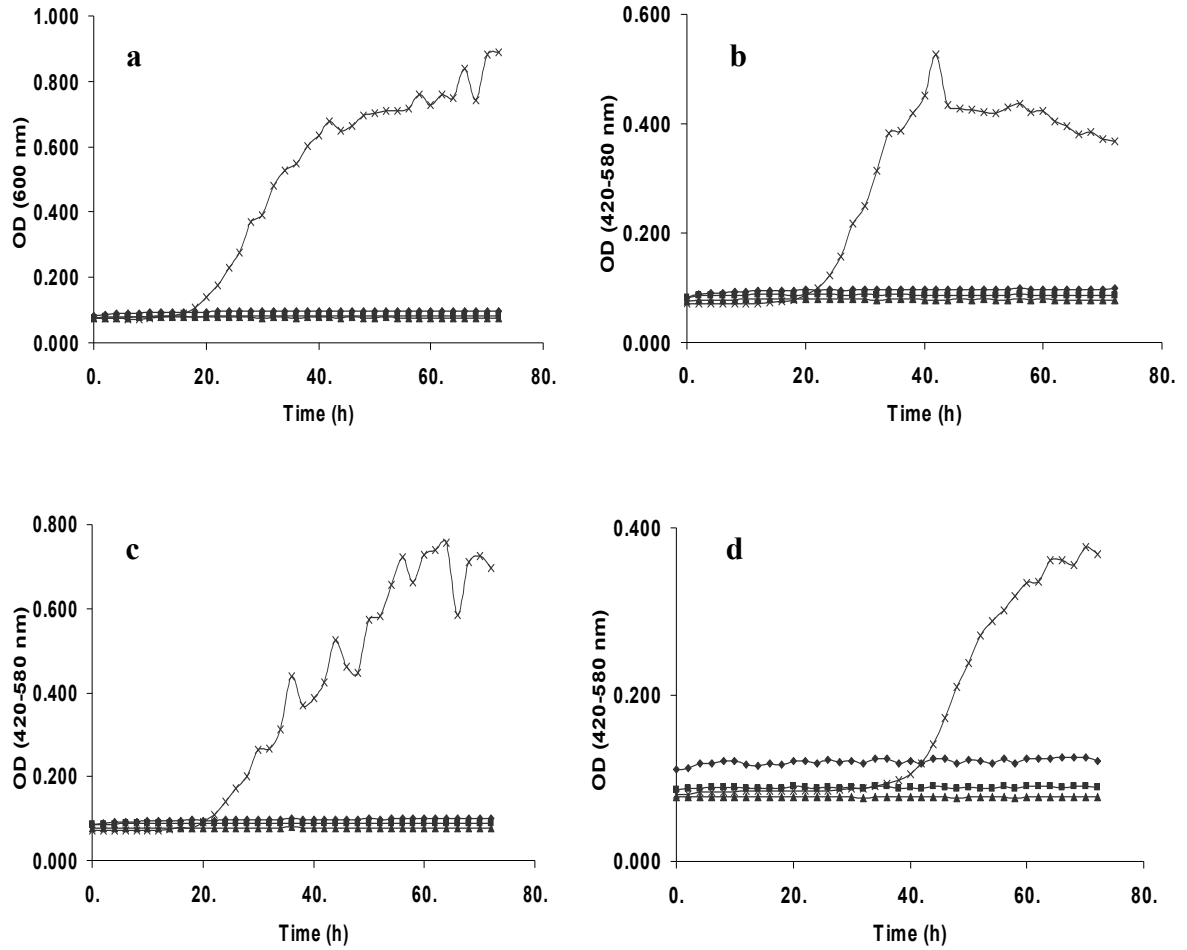

Figure 2. Antibacterial activity of the ACN-extract of the ascidian *Synoicum pulmonaria* against the Gram-negative bacteria *E. coli* (a) and *L. anguillarum* (b) and the Gram-positive bacteria *C. glutanicum* (c) and *S. aureus* (d). The figures show the optical density which was measured at 420-580 nm in a bacterial suspension of 5×10^3 cells per well containing bacteria alone (x) or bacteria with ACN-extract at a concentration of 0.02 mg/ml (▲), 0.04 mg/ml (■) and 0.08 mg/ml (♦).

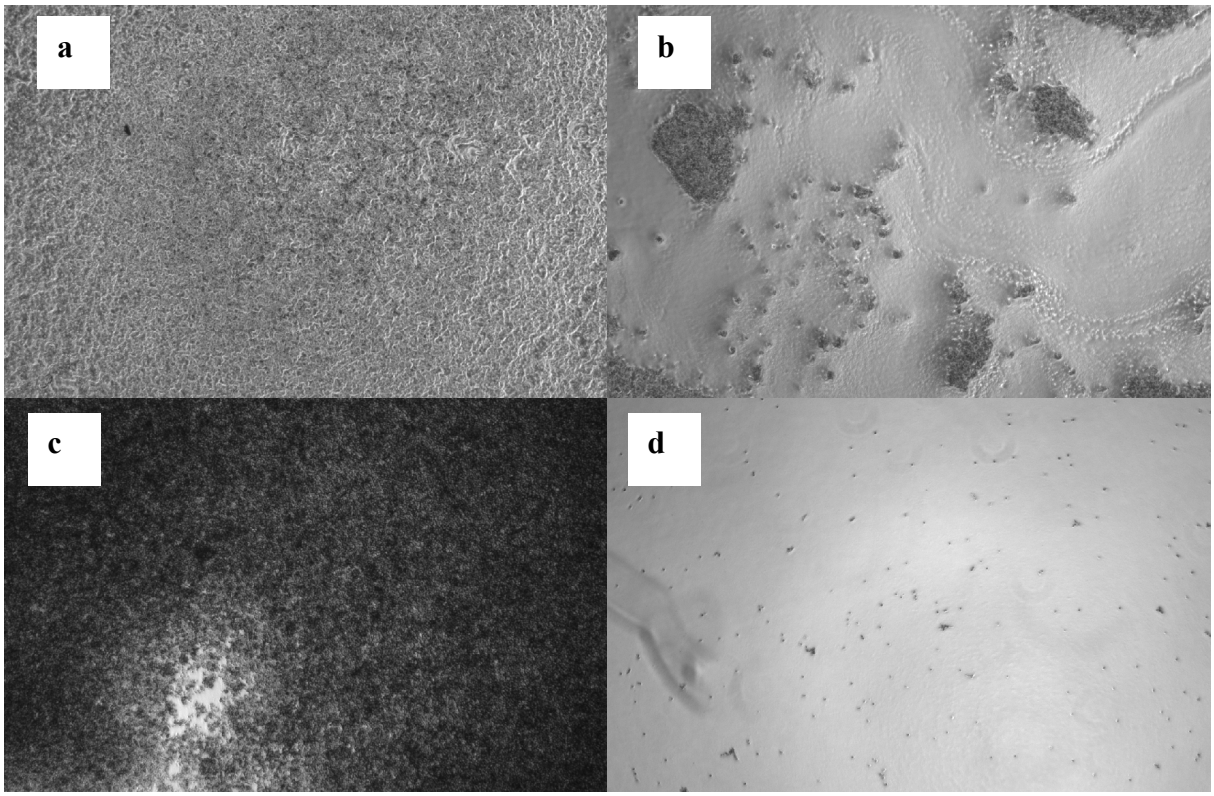

Figure 3. Antifungal activity of the ACN-extract of the ascidian *Synoicum pulmonaria*. (a) Growth of *C. albicans* without the ACN-extract. (b) Growth of *C. albicans* containing 0.04 mg/ml of the ACN-extract. (c) Growth of *S. cerevisiae* without the ACN-extract. (d) Growth of *S. cerevisiae* containing 0.02 mg/ml of the ACN-extract. The fungal spores and the ACN-extracts were inoculated in 96-well microtitre plates for 48 h before pictures were taken using an Axiovert 40 CFL microscope with an AxioCam MRm camera from Zeiss (50x magnification).

Figure 2 a)

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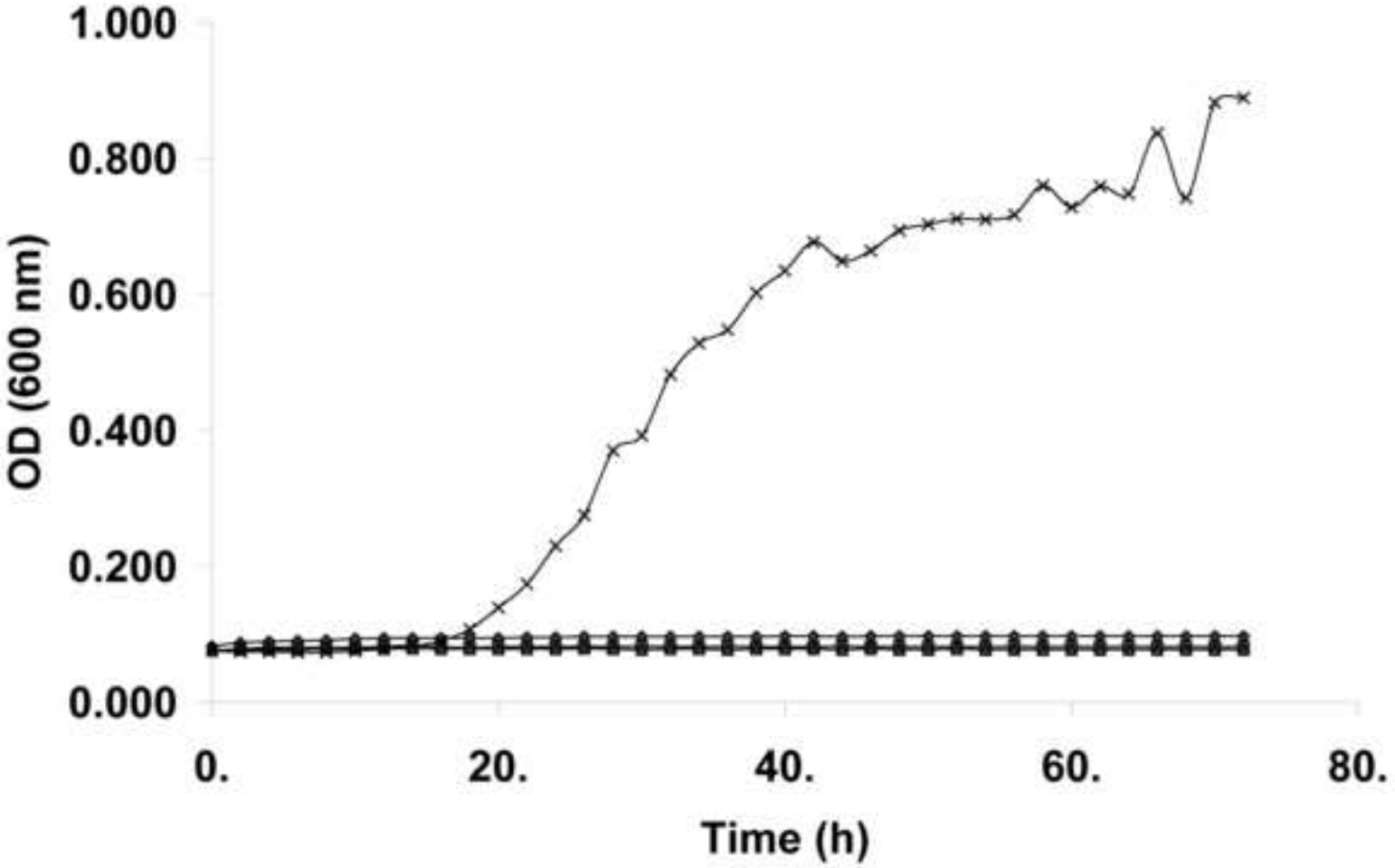

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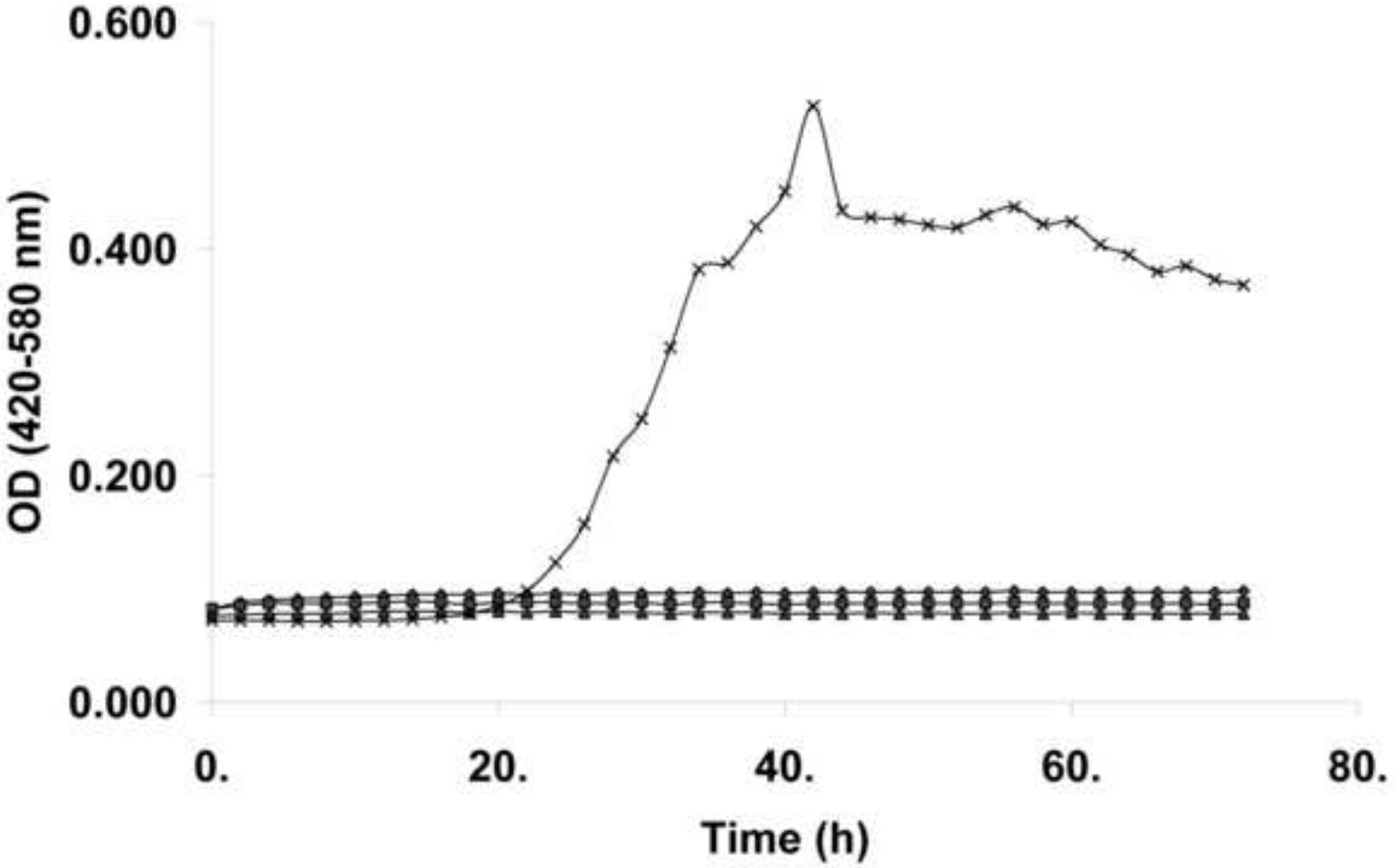

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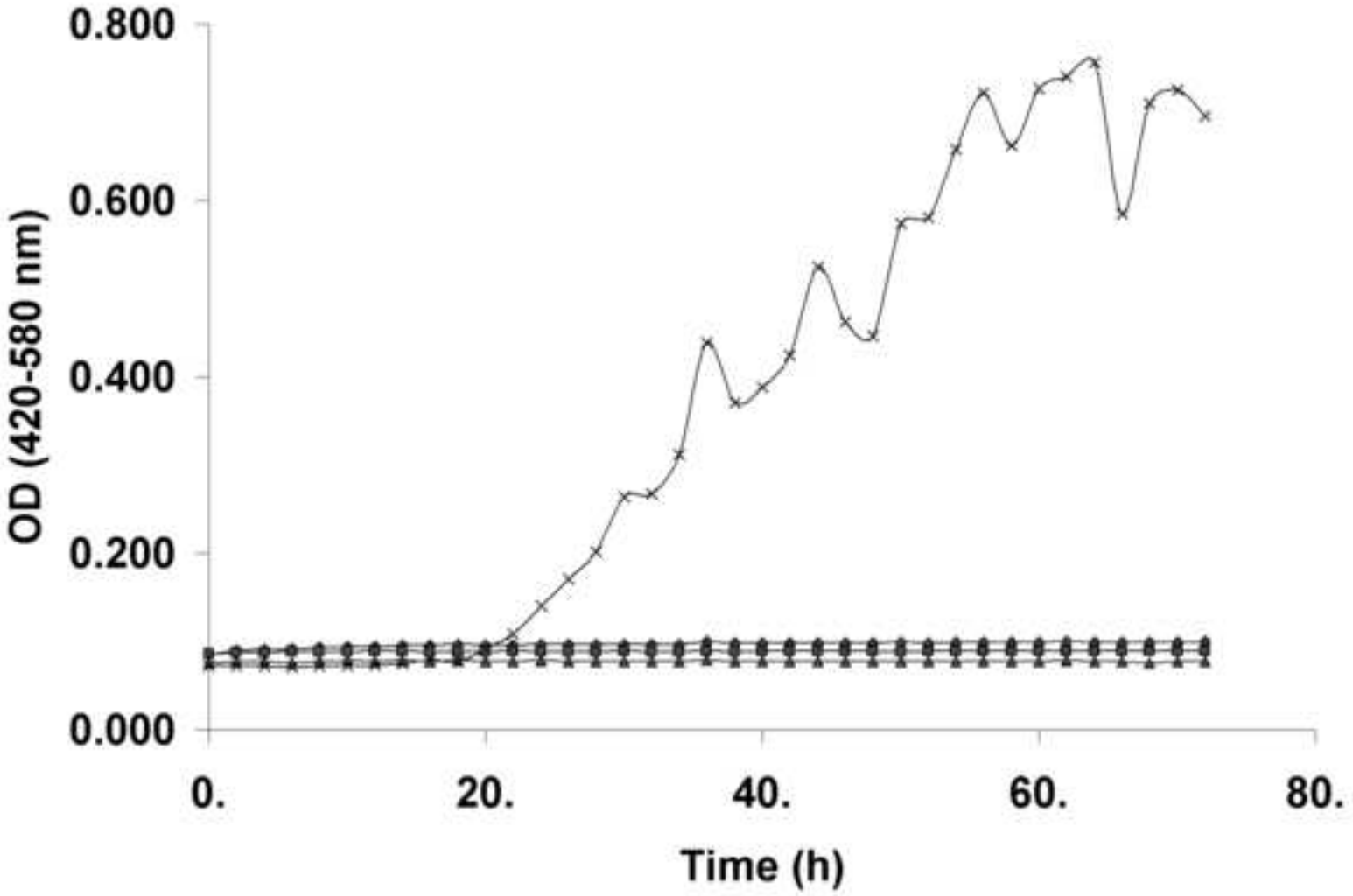

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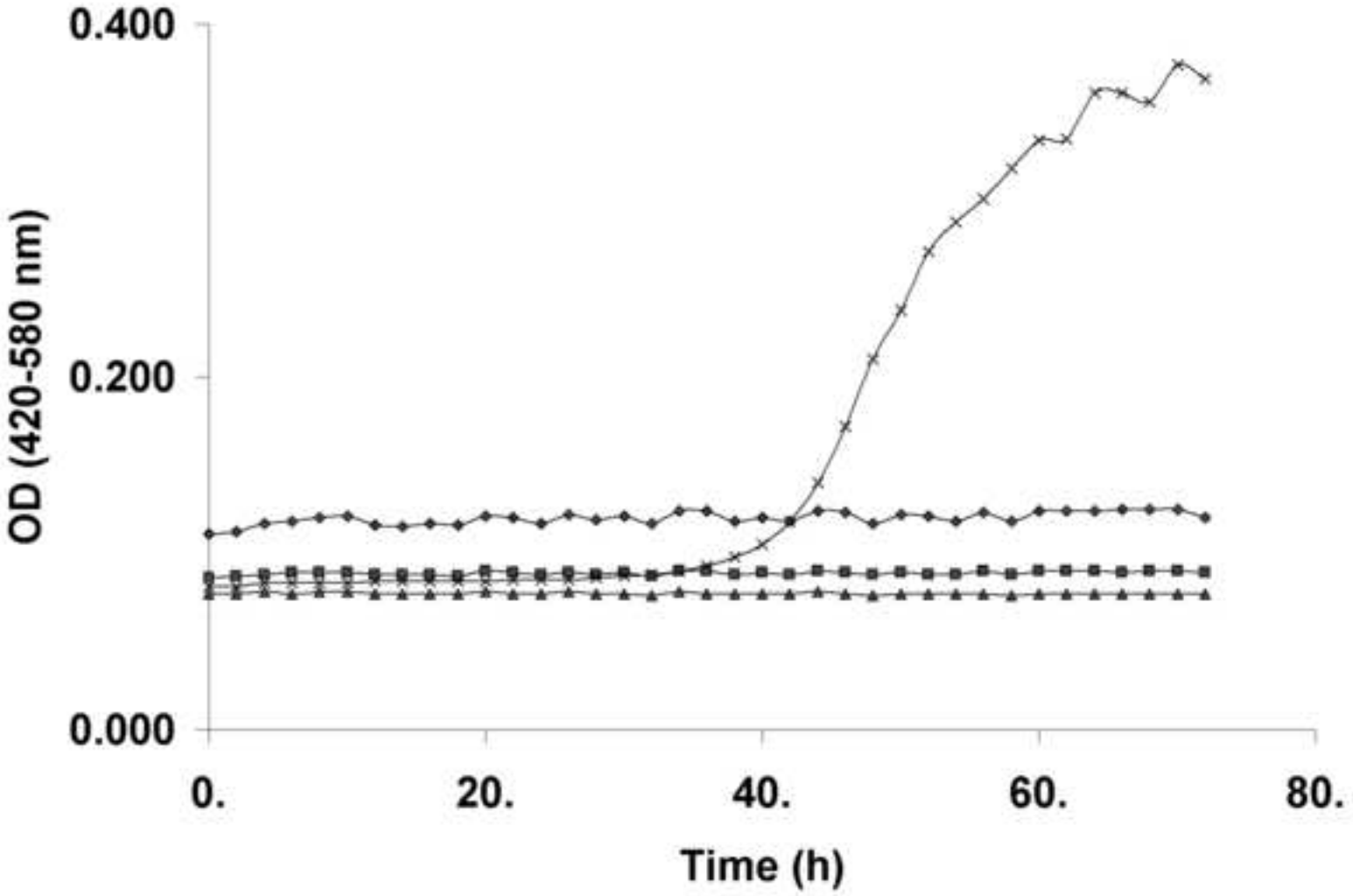

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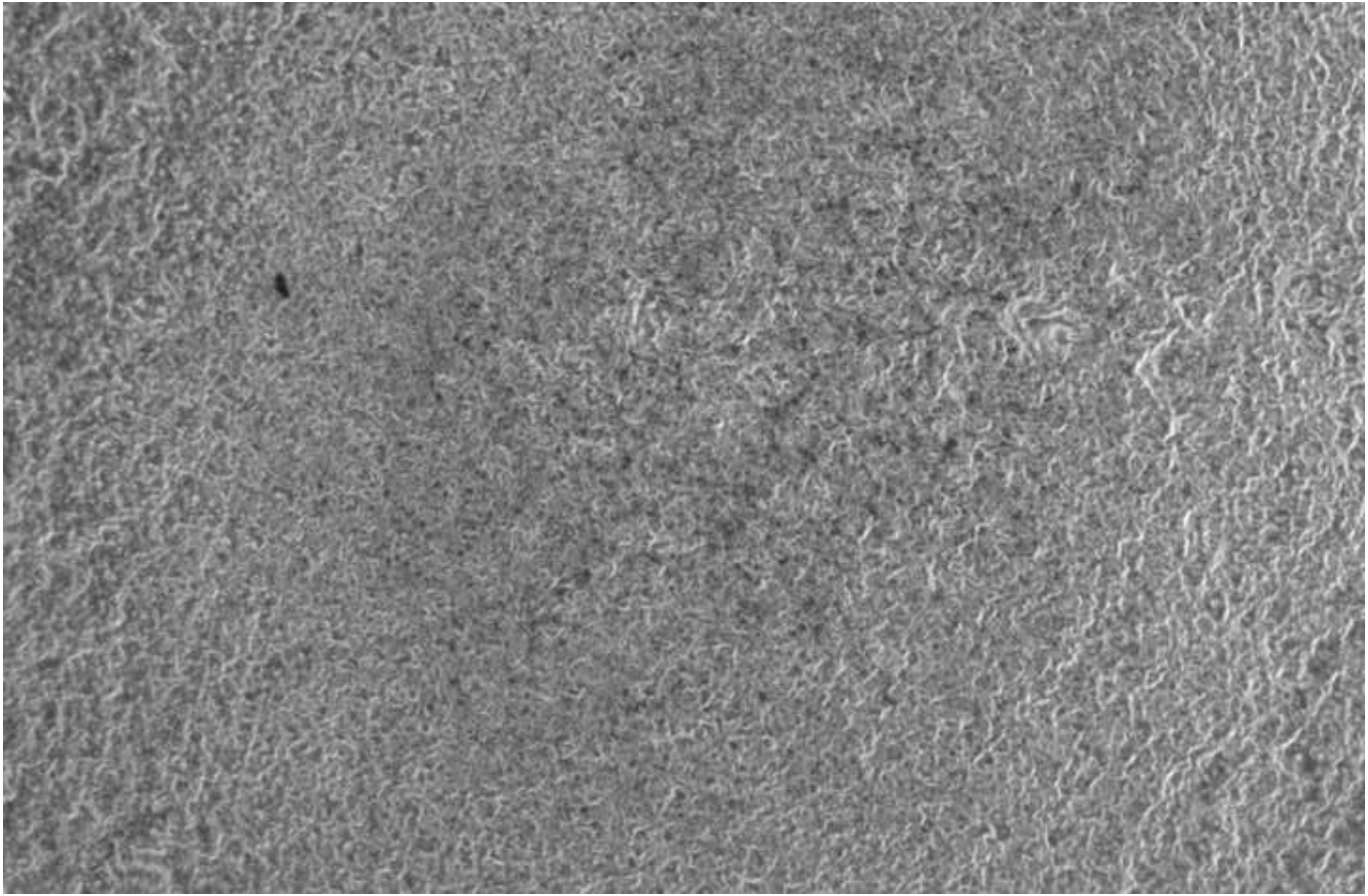

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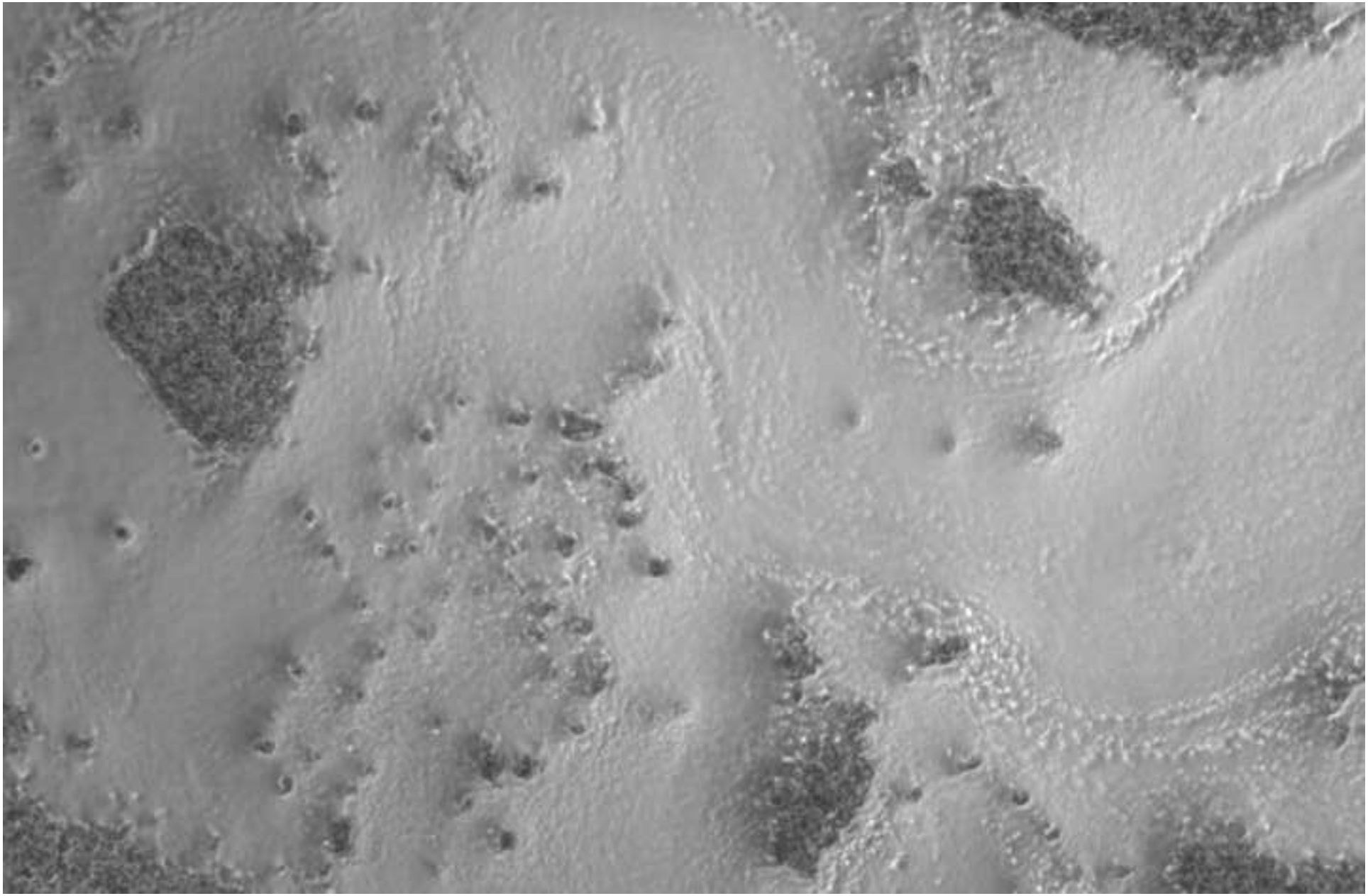

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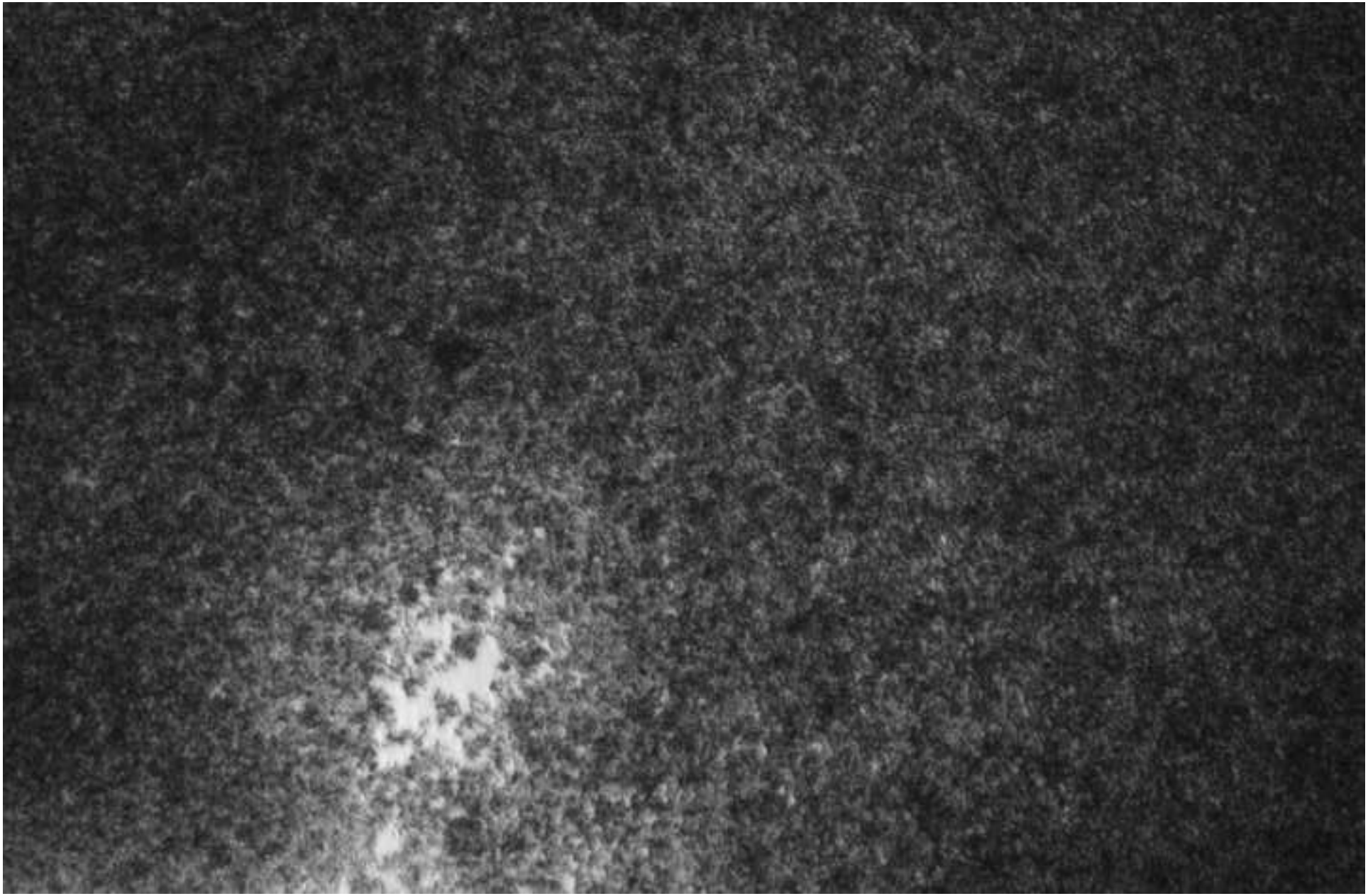

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