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# Estimate of microbial biodiversity in Electra pilosa and Alcyonium digitatum

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Abstract

In attempting to characterize the microbial population of the marine species *Electra pilosa* and *Alcyonium digitatum* this study yielded a wide range of microbial growth using *in vivo* cultivation techniques on agar plates and PCR. The methods of the study were evaluated to the benefit of coming studies.



Keywords

*Electra pilosa*, *Alcyonium digitatum*, PCR, agar cultivation, marine organisms



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# Estimate of microbial biodiversity in *Electra pilosa* and *Alcyonium digitatum*

Hélène Harnemark


## Sammanfattning

Våra världshav är en relativt ny källa för vetenskapliga upptäckter. Det har länge varit svårt att utnyttja och undersöka något på havets bottnar. När resurser och utrustning under de senaste årtiondena blivit bättre har vi sett att här finns mycket att finna.

Man har exempelvis hittat havslevande djur som kan skydda sig från parasiter utan att ha ett immunförsvar. De har tagit hjälp av bakterier som tillverkar olika giftiga ämnen som sprids i djurets omgivningar eller stannar på dess yta, vilket ger ett skydd från vissa rovdjur. Detta fenomen är både av biologiskt intresse och kan vara ett sätt att hitta nya bekämpningsmedel och läkemedel.

Taggig tångbark, *Electra pilosa*, är ett exempel där man sett att det finns ett skydd från rovdjur samtidigt som man hittat bakterier inuti djuret. Man har ännu inte funnit vad som bidrar till detta skydd eller vilka bakterier det handlar om. Död mans hand, *Alcyonium digitatum*, är välkänd, men har tidigare inte varit känd att vara i symbios med bakterier. Då den finns lättillgänglig är den väl värd att undersöka.

Denna studie har funnit att det troligen finns ett stort antal bakteriestammar i både *E. pilosa* och *A. digitatum*. Odlingar från djuren gav ett spektra av bakterier. Möjligen finns det hos någon av dessa en intressant produkt att undersöka vidare.



**Examensarbete 20 p inom Civilingenjörsprogrammet i molekylär bioteknik**

**Uppsala universitet, december 2006**

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# Introduction

## 1. Aims of the study

This study attempted to characterize the microbial population of two marine eukaryotic organisms, *Electra pilosa* and *Alcyonium digitatum*. The methods involved were evaluated and optimized for these species.

### 1.1. Finding the inhabitants

In many marine eukaryotes a number of prokaryotes can be found.[1] The spectra of microbial diversity differ from one eukaryotic species to another. In the case of *E. pilosa* at least four bacterial genera has been found.[1] This study aims at painting a more detailed picture of the inhabitants of *E. pilosa*.

*A. digitatum* has not been widely studied from a biodiversity point of view. This study will therefore hopefully shed some light on one aspect of this species internal affair.

### 1.2. Evaluating the techniques

Techniques used to grow marine bacteria in conditions suitable for humans to work in are not thoroughly investigated. Therefore an evaluation of the different media used and of the DNA preparation protocols is much needed for bringing this field of science forward. Only a very limited number of laboratory growth media for growing marine bacteria have been developed.

## 2. Bacterial communities in marine organisms

Studies have shown a multitude of marine eukaryotes hosting bacterial communities in some way or another: in tissues; on the surface; in the direct surroundings outside the eukaryote. In many ways these bacterial groups seem to present the eukaryote with competitive advantages, a symbiotic relationship.[1,2,3,4,5,6]

This symbiosis is thought to be associated with eukaryote health in many cases although how has not yet been thoroughly verified but nutritional and protectional aspects are the main possibilities.[4,5,6] It is very likely that some more dominant bacterial groups are pathogenic rather than beneficial for the eukaryote.[2] In the literature, there are examples of both pathogenic and beneficial bacterial inhabitants of marine eukaryotes.[1]

Isolated bacteria have many times been shown to be species specific[3] or specific to families or specific types of eukaryotes.[2,6] In some investigated marine organisms there have been bacteria with significant toxicity which has been proposed to live in symbiosis with the host eukaryote and therefore giving the whole eukaryote a toxicity aimed at its predators.[6] This symbiosis may also correspond with the absence of pathogenic bacteria on the surface of the eukaryote. Such an arrangement would not only provide protection for

the bacteria but also a certain degree of protection from parasitic organisms for the eukaryote. [8]

Such findings may also be valuable for research and development of environmentally friendly compounds for humans to use both in medicine and in other aspects of life i.e. paints and chemicals.[7] A significant example of this is found in the marine sponge *Geodia baretti* which hosts a large number of different prokaryotes.[8] *G. baretti* produces the antibacterial and antiviral alkaloid barettin.[9,10] Barettin has recently been shown to have antifouling properties, *i.e.* the presence of barettin inhibits the settlement and growth of barnacles (*Balanus improvisus*).[11] This discovery could potentially lead to the development of more ecologically sound methods of preventing barnacles from growing on boat hulls. It is not yet known whether *G. baretti* itself produces barettin or if one (or several) of the numerous prokaryotes living in it are responsible for the production.

## 3. Marine species used in study

In this study two different marine eukaryotic species were studied, the soft coral *Alcyonium digitatum* (Dead Man's Fingers) and the moss animal *Electra pilosa* (Hairy Sea-mat). Both can be found in Swedish salt water.

### 3.1 *Electra pilosa*

*Electra pilosa*, or Hairy Sea-mat, is a small marine moss animal (*Bryozoa*) living on algae, cliffs, stones and shells. In Swedish waters it lives along the west coast and the southern coast of Skåne, to a depth of approximately 70 meters. The animal itself is very small, approximately 0.5 mm in diameter, and resembles a small, whitish ring on the surface they live on. Hairy Sea-mat form large colonies that are up to 10 cm in diameter. Larger colonies grow in a very characteristic star-shaped way, at which point they can easily be recognized. The colonies are very thin, so thin it sometimes is hard to distinguish them looking from the side. Despite being so small, they are quite hard, caused by calciferous deposits in the walls of the single animals.[12]

Like other moss animals, Hairy Sea-mat lives on particles and plankton floating in the water surrounding it. There are very few natural predators of Hairy Sea-mats, but among them are sea spiders and sea urchins.[12]

It has been shown that *E. pilosa* contains some kind of bacterial communities[1], and this was a decisive factor on why this organism was included in the study. Previous studies have shown that the bacteria are contained inside *E. pilosa* and not on the surface and this is indeed an interesting aspect to work with. Most sea living eukaryotes have parasites and bacteria on the surface and those who can protect themselves from that may have an interesting way of doing just that. Since *E. pilosa* is one of those which has very few parasites and predators on its surface that was another reason to include it in this study.

## 3.2 *Alcyonium digitatum*

*Figure 1: A. digitatum*, Dead Man's Fingers. Picture from http://www.biopix.dk, with permission.

Also known as Dead Man's Fingers, *Alcyonium digitatum* is a very common soft coral living in almost the entire northeast Atlantic Ocean, to a depth of up to 100 meters. The colonies closely resemble a decomposing human hand, hence its name. In colour it varies from almost white to completely orange. It has a rather fuzzy appearance, mostly due to the fact that the polyps of the single animals protrude all over the surface (figure 1).[13]

*A. digitatum*, like most soft corals, lives on plankton, primarily zooplankton.[14] Several species, such as sea snails and nudibranches, predate on Dead Man's Fingers.[13]

*A. digitatum* has not previously been investigated whether it contains prokaryotic symbionts and therefore it was an interesting organism for this study.

# Methods and materials

## 1. Outline of study methodology

This study was divided into two assays, a marine microbial cultivation assay and a microbial DNA-extraction from eukaryote tissue assay. Both assays made use of the same species, *Electra pilosa* and *Alcyonium digitatum*, and the primers used in the PCR were identical. In the marine microbial cultivation assay the two species were grown on agar plates of different types and analyzed with ocular-, microscopic- and PCR-techniques.

The microbial DNA-extraction from eukaryote tissue assay was very similar to the marine microbial cultivation assay, simply excluding the growth of bacteria. Both assays were supposed to be concluded with sequencing of the PCR-products and a phylogenetic analysis. This will be done shortly, but will not be included in this study.

## 2. Marine microbial cultivation assay

The main objective of this assay was to obtain cultures of marine bacteria on agar plates. These bacterial cultures would not only yield sufficient amount of material for DNA extraction and sequencing but would also provide a spectrum of live marine bacteria for further studies. Furthermore a visual classification could be done in parallel to the DNA extraction and sequencing and hence give a more reliable phylogenetic result.

### 2.1. Origin of eukaryote material

The eukaryotes used in the marine microbial cultivation assay, *E. pilosa* and *A. digitatum*, were collected in the Swedish Gullmarsfjorden, south/southeast of Lysekil. The sample eukaryotes were kept at 4˚C in seawater and transported to Uppsala University within 48 hours. Further storage was at 4˚C in seawater. The following preparations of the eukaryotes were done within 36 hours.

### 2.2. Cultivation media

The marine bacteria were grown on a set of eight (8) different agar media: M1, M3-M9. These media were solid plated media used to obtain as many different kinds of bacteria as possible with respect to different needs in nutrition and salt levels that might be relevant for novel marine bacteria. All except one media (M1) contained antibiotics for inhibition of both fungi and contaminating non-marine bacteria as well as for a deliberate inhibition of different, though unknown, marine bacteria. The antibiotics used were chosen according to the results of previous studies.[5,7] This was done for the single purpose of

getting the widest bacterial spectra possible, *i.e.* inhibiting some bacteria on one media while hopefully promoting the same on another media.

The antibiotics used were cycloheximide[5,7] and nystatin[5] for inhibition of fungal growth as well as nalidixic acid[5], rifampicin[7] and novobiocin[7] for inhibition of bacteria.

M1 was based on marine agar (Difco) containing salts and minerals imitating sea water levels. M3 was based on actinomycete isolation agar (Difco) and also containing low levels of the antibiotics nalidixic acid (Sigma-Aldrich), cycloheximide (Riedel-de Haën) and nystatin (Sigma). The actinomycete isolation agar is an agar promoting the growth of often slow growing actinomycetes.

The remaining six (6) agar plates were all based on bacto agar (Difco) which is an agar used for growth in most basic projects involving for instance bacteria or yeast. Media M4 and M5 additionally contained the same low levels of antibiotics as did M3, *i.e.* nalidixic acid, cycloheximide and nystatin. M4 used small amounts of starch (Kebo Lab AB) as nutrients and also the salts $K_2HPO_4$ and NaCl. M5 used both yeast extract (Merck), malt extract (Fluka) and dextrose (Sigma) as nutrients as well as the salt NaCl.

The M6 media contained starch, yeast extract and peptone (Sigma) as nutrients and a single antibiotic, cycloheximide, in a ten times larger amount than in any other media. M7 used very low levels of peptone and mannitol (Fluka) as nutrients and the antibiotics rifampicin (Sigma) and cycloheximide. M8 and M9 had no additional nutrients and two antibiotics: novobiocin (Riedel-de Haën) and cycloheximide in M8; rifampicin and cycloheximide in M9.

Two of these solid plated media, M1 and M3, were prepared with water. The rest, M4-M9, were prepared with, filtered, seawater to obtain the optimal salt level for marine bacteria.

The media was prepared in glass bottles, autoclaved for 15 min in 121˚C and 1.1 bar. Thermally unstable (sterile) ingredients were added to each bottle when the temperature of the media had reached approximately 55˚C. Aliquots of 40 mL were poured onto sterile plastic plates and lidded. The plates were kept in room temperature for 24 to 48 hours to dry and solidify. If the plates were not used immediately after 24 to 48 hours they were stored in 4˚C until further use. See appendix 1 for details regarding media composition.

### 2.3. Bacterial growth and incubation

Since the two different eukaryotes used in this project were of a very different kind, morphologically and in size, two different approaches were used in extracting tissue and sterilizing it before cultivating the bacterial inhabitants of the extracted tissues.

In the case of *A. digitatum* this process was done by cutting out a 1 $cm^3$ cube with sterile scalpels from the inner part of the eukaryote. This small piece of tissue was then rinsed in 70% ethanol and immediately transferred into sterile water. The tissue was then mixed with 9 mL of sterile water and thoroughly mixed in a homogeniser (YellowLine DI 25 Basic) producing a finely cut “tissue-broth”. This tissue mixture was diluted stepwise from the concentration 1/10 to 1/10000 producing four solutions to be used for cultivation of bacteria on agar plates, A.d.1/10, A.d.1/100, A.d.1/1000 and A.d.1/10000. This nomenclature was also adopted for identification of the plates.

For *E. pilosa* the sterility of the procedure was much more difficult to maintain. The whole organism of *E. pilosa* is growing flatly on seaweed and it is also very small. Because of this, extracting an internal part of tissue is not conceivable without much more complex instruments than was available in this project. Therefore, the seaweed was made sterile by rinsing it in sterile water and 70% ethanol. *E. pilosa* was then carefully removed with a sterile scalpel, weighted and then put in 1 mL of sterile water. This mixture was also thoroughly mixed (YellowLine DI 25 Basic) and diluted three times, in the same way as *A. digitatum*, producing four solutions to be used in the cultivation assay. Solutions and plates with corresponding solution were named E.p.1, E.p.2, E.p.3 and E.p.4 with E.p.1 being the one with highest concentration.

As controls, the seaweed and a small shell living in abundance on the seaweed and very close to *E. pilosa*, were used. Both tissues were extracted in the same fashion as *E. pilosa*. The only difference was that these samples were not diluted but spread in only one concentration onto plates. These samples and the plates corresponding with them are named "alg" and "shell". A sample of the sterile water from the tissue extractions were used as contamination control.

Eleven (11) different solutions were used on eight different kinds of media, making a total of 88 plates (11 x 8). These initial plates were cultivated in 22˚C for up to six weeks. Every second day they were checked for new colonies and if any such appeared these were transferred and spread on fresh plates with the same media.

This procedure was redone as many times as were needed to produce pure cultures of bacteria, which most often took 3-4 restreakings. When pure lines were obtained and not immediately used in the next step of the project they were stored in 4˚C.

## 2.4. DNA-extraction

Colonies were classified by their colour, texture and size, visually and in 100 times magnification. Every media and every species was checked separately. From each colony type found, one colony was taken with a sterile stick and resolved in 3 mL of marine broth (Difco), a liquid media with salts, nutrition and minerals imitating sea levels. This solution was then incubated on a shaker in 22˚C for 36 to 72 hours depending on the growth speed of the plated colony. The slower the growth was on solid media, the longer the incubation time in liquid media was.

DNA from each incubated colony sample was then extracted by a simple protocol. Initially 1 mL of incubated solution was transferred to 1.5 mL tubes (Eppendorf) and spun down (5 min, 13000 rpm) in a centrifuge (Biofuge, Fesco). The marine broth was then discarded and the cells were washed with 1 mL sterile water. The cells were then again spun down (10 min, 13000 rpm) and the water removed.

The cells were resuspended in 400µL TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, sterilized). 100 µL lysis buffer (12.5 M Tris-base, 0.25 M EDTA, 12.5 mL 20 % SDS per litre $dH_2O$, pH 7.4, sterilized) were then added and the tubes were briefly vortexed and thereafter incubated for 30 minutes at 65˚C.

125 µL KAc (5 M) was added and the tubes were vortexed and incubated for 1 hour on ice. The tubes were then spun for 15 minutes in 13000 rpm and 400 µL aliquots of the

supernatant was collected to two new tubes, making a duplicate. The DNA was then precipitated with 40 µL NaAc (3 M) and 1 mL ice cold ethanol 95 %. The tubes were mixed by inverting them a few times and then they were spun down (5 min, 13000 rpm). The supernatant was then collected and washed with ethanol 70 % and respun shortly again. The ethanol was removed and the pellets were left to dry.

When completely dry, the pellets were suspended in 100 µL $dH_2O$. The extracted DNA was stored in 4˚C after being checked for DNA content on a 1 % agarose gel (gel electrophoresis).

### 2.5. PCR analysis

For identification of the marine bacteria in this study the 16S ribosomal RNA gene of *E. coli* was used. This gene has been very useful in various phylogenetic studies involving bacteria giving us useful phylogenetic data to compare any novel bacterial species with and thus we have the means to a basic identification of our finds. In this part of the genome there is what is called a hyper variable region, V3, which has been used to identify novel bacterial species in other marine environments.[15] The primers used in this project produce a 194 bp sequence from position 338 to 536 in 16S rRNA of *E. coli*. The reverse primer consisted of 18 bases, 5'-GWATTACCGCGGCKGCTG-3', and the forward primer consisted of 20 bases, 5'-ACTCCTACGGGAGGCAGCAG-3'. All primers were ordered from Invitrogen.

Every PCR made had three controls including two negative controls, one lacking DNA template and the other enzyme for the reaction. Control number three was a positive one with *E. coli* as template. Every bacterial sample was made in duplicate to eliminate misinterpretations of the result.

For the reaction a premade mixture, Platinum PCR SuperMix (Invitrogen), was used as buffer and polymerase. This mixture includes anti-*Taq* DNA polymerase antibody, $Mg^{++}$, dNTPs and recombinant *Taq* DNA polymerase. To every reaction tube 0.5 µM of each primer (0.5 µL), 4 µL of template (2 µL in the positive control) and 45 µL of Platinum PCR SuperMix was added (47 µL in the positive control), making a total volume of 50 µL. In the negative control reactions the lacking ingredient was substituted by sterile water in the correct amount. The reaction tubes were kept on ice until put in the PCR machine.

The PCR was run with the lid heated to 100˚C to prevent uneven temperatures in the tubes and condensation in the lids of the tubes. The tubes were preheated to 95˚C for 10 minutes in the PCR machine. Thereafter 30 cycles were run, with denaturation for 1 minute in 94˚C, annealing for 30 seconds in 53˚C and extension for 1 minute in 72˚C. Finally the reaction was halted by cooling the samples and tubes to 4˚C. Tubes were stored in 4˚C until the PCR products could be analysed in agarose gel electrophoresis.

## 3. Microbial DNA-extraction from eukaryote tissue assay

In this part of the study the objective was to see if a simplified assay including only DNA-extraction and PCR would yield the same results as the more complex assay described

above in section 2 of materials and methods. It was also investigated whether lyophilized material yielded DNA in the same amounts as live material when extracted.

### 3.1. Species and origin of samples

*Electra pilosa* was used in this assay. The samples were not purified or sterilized before being lyophilized any more than collecting them and having the species confirmed. The organism was collected in the Swedish Koster Fjord, 1 nautical mile from Tjärnö Marine Biological Laboratory (58˚53'N, 11˚8'E). The samples were lyophilized and stored in airtight glass bottles in freezer until further use.

### 3.2. DNA-extraction

To extract DNA from the lyophilized tissue of *E. pilosa* 0.10 g tissue was homogenized and mixed with 1.5 mL of extraction buffer with SDS (100 mM EDTA, 100 mM TRIS, 100 mM sodium phosphate, 1.5 M NaCl, 1% SDS, pH 8.0). The homogenization and extraction of the samples were done in duplicates.

The mixture was incubated for 2 hours with occasional shaking. Isolation of DNA was done with one volume of dichloromethane/isoamylalcohol in parts 24:1 and incubated for 30 minutes, shaking, in room temperature. The samples were then spun down (15 min, 13000 rpm). The water phase was carefully collected and mixed gently with 0.6 volume of isopropanol. The sample was incubated over night in -20 ˚C.

The mixture was then spun at 13000 rpm for 10 minutes and the supernatant was discarded. The pellet was washed with ethanol 70 % and the tubes were spun down and the ethanol discarded.

If the pellet after this step looked virtually clean, *i.e.* no discoloration or with obvious contamination, the pellet was dried. If the pellet was not pure enough, then it was resuspended in 1 mL of TRIS (100 mM) and the whole procedure from the isolation with dichloromethane/isoamylalcohol was repeated.

The samples were checked on 1 % agarose gel (gel electrophoresis) to ensure DNA had been extracted and stored in -20 ˚C.

### 3.3. PCR analysis of lyophilized samples

The PCR was done using the same protocol and the same controls as the PCR described in section 2.5 of materials and methods.

# Results

## 1. Cultivation assay

### 1.1. Growth of bacteria

Initially there was major growth after 12-18 hours of incubation on some of the plates with the highest concentration of applied eukaryote tissue from both *A. digitatum* and *E. pilosa i.e.* A.d.1/10 and E.p.1. Some growth was also seen on a few plates, from both species, with lower concentrations and even on those with the lowest concentration of *A. digitatum*. Both the shell and the algae plates had minor growth at this time.

The growth on the plates with high concentration, A.d. 1/10, E.p. 1, Shell and Alg, were very rapid, resulting in overgrowth which soon rendered the plates unusable. A.d. 1/100, A.d. 1/1000, E.p. 2 and E.p. 3 proved to be much easier to handle, and the growth were moderate, giving a wide range of colony types, which didn't need as many restreakings as those from the high concentration plates did. The plates with the lowest concentration, A.d. 1/10000 and E.p. 4, had minor growth or none, for a long time.

*Table 1.* Number of unique colonies isolated on different media

| | E.p.1 | E.p.2 | E.p.3 | E.p.4 | A.d. 1/10 | A.d. 1/100 | A.d. 1/1000 | A.d. 1/10000 | Shell | Algae | Water |
|---|---|---|---|---|---|---|---|---|---|---|---|
| M1 | | 2 | 1 | | | | 4 | 1 | 2 | 8 | |
| M3 | 8 | | | | 1 | 1 | 2 | | 6 | 2 | |
| M4 | 4 | | | | 1 | 1 | | | | | 2 |
| M5 | 9 | | | 2 | 7 | 1 | 1 | | 2 | 4 | |
| M6 | | 3 | 10 | 2 | | | 4 | 5 | 1 | 11 | 2 |
| M7 | 1 | | | | 2 | | | | | 1 | |
| M8 | 4 | | | | 4 | 3 | | | 1 | 2 | |
| M9 | | | | | | | | | 2 | 1 | |

When plated, the colonies had a very varied speed of growth and this resulted in many colonies growing into each other. When colonies were respread until each type of colony on each separate type of plate had a unique identity, as far as can be said without genetic control of the result, a wide range of bacteria could be seen. All plate types yielded unique colonies, M6 with the most (38), M9 with the fewest (3). Colony type count of the other plates were M1 (18), M3 (20), M4 (8), M5 (26), M7 (4) and M8 (14). This makes a total of 131 unique colony types, even though some most likely are identical due to growth on different plate types making it difficult to say whether they are different types or identical. See table 1.

When looking at the eukaryote origin, it was seen that a total of 46 colony types had *E. pilosa* origin and 38 had *A. digitatum* origin. The numbers from the controls are smaller, with 12 of shell origin, 28 of algae (alg) origin and only 4 of water origin. See table 1 and table 2.

*Table 2.* Microbial diversity on the investigated media

| | Number of different colonies | PCR products |
|---|---|---|
| M1 | 18 | |
| M3 | 20 | |
| M4 | 8 | 3 (E.p. 1; colonies 27, 28 and 29) |
| M5 | 26 | 1 (A.d. 1/10; colony 6) |
| M6 | 38 | 2 (E.p.2 ; colonies 13 and 14) |
| M7 | 4 | |
| M8 | 14 | 2 (E.p.1; colonies 33 and 34) |
| | | 3 (A.d. 1/10; colonies 35, 36 and 38) |
| | | 3 (A.d. 1/100; colonies 39, 40 and 41) |
| | | 1 (algae; colony 42) |
| M9 | 3 | |

1.2. Plates

The plates that yielded the most amount of growth were M1, M5 and M6. Many colonies on M1 were of the same type, which was not the case with M5 and M6. M9 appeared the most unfriendly habitat for any bacteria with very few colony types growing, and those which did grow, grew very slowly.

1.3. Contamination

Fungal contamination occurred after approximately 8 days on a few plates. These plates were not studied further in the project. Fungi appeared on a few plates all through the study and those plates were carefully set apart from the rest to minimize further contamination.

After approximately 8 days the control plate (with water) of M6 had growth. No similar colonies could be seen on the other M6 plates. The M1 plate control was likewise contaminated after 15 days of incubation but as with M6 no similar colonies could be detected on other M1 plates. After 22 days of incubation there were signs of contamination on the control plates of M1, M4 and M7. The first set of plates was incubated and handled until contaminated or no growth had appeared after 6 weeks.

1.4. PCR products

Not all colony types, due to time limitations, were transferred to liquid media growth and PCR, but from those who were, almost all yielded PCR products. Most of these needed an annealing temperature of 53˚C to yield.

PCR products are confirmed from one colony on M5 plates, A.d.1/10 (colony named 6), two colonies from M6 plates, E.p.2 (colonies named 13 and 14), three colonies from M4 plates, E.p.1 (colonies named 27, 28 and 29) and nine colonies from M8 plates, E.p.1 (colonies named 33 and 34), A.d.1/10 (colonies named 35, 36 and 38), A.d.1/100 (colonies named 39, 40 and 41), alg (colony named 42). In total fifteen PCR products can be sent for sequencing from this part of the study. See table 3.

*Table 3*. Isolated microorganism

| Colony name | Origin |
|---|---|
| 1 | *Electra pilosa* dilution 1; Media M4; colony 27 |
| 2 | *Electra pilosa* dilution 1; Media M4;; colony 28 |
| 3 | *Electra pilosa* dilution 1; Media M4;colony 29 |
| 4 | *Electra pilosa* dilution 2; ; Media M6; colony 13 |
| 5 | *Electra pilosa* dilution 2; ; Media M6; colony 14 |
| 6 | *Electra pilosa* dilution 1; ; Media M8; colony 33 |
| 7 | *Electra pilosa* dilution 1; ; Media M8; colony 34 |
| 8 | *Alcyonium digitatum* dilution 1; Media M5; colony 6 |
| 9 | *Alcyonium digitatum* dilution 1/10; Media M8; colony 35 |
| 10 | *Alcyonium digitatum* dilution 1/10; Media M8; colony 36 |
| 11 | *Alcyonium digitatum* dilution 1/10; Media M8; colony 38 |
| 12 | *Alcyonium digitatum* dilution 1/100; Media M8; colony 39 |
| 13 | *Alcyonium digitatum* dilution 1/100; Media M8; colony 40 |
| 14 | *Alcyonium digitatum* dilution 1/100; Media M8; colony 41 |
| 15 | Algae, non-diluted; Media M8; colony 42 |

## 2. DNA-extraction assay

PCR products were confirmed from *E. pilosa*. No further steps were made during the time of this study.

# Discussion

## 1. Range of species

### 1.1. *Electra pilosa* origin

Since there was not enough time to sequence the obtained PCR products from the marine eukaryotes the only tool available was to group the bacteria by eye and microscope. A multitude of different bacteria were identified visually. Since no sequencing took place, there is very limited information on real contamination available. It is possible, although not probable, that a number of the colonies seen were from the same bacterial strain or, more likely, from the laboratorial surroundings. This aspect can, with the results this far, not be evaluated further.

In the case of *E. pilosa*, it can also be argued that the bacterial flora was transferred to the laborative environment and all growth there, was indeed a mixture of the three eukaryotes, *E. pilosa*, the shell and the algae that the first two grew on. This is of course something that only can be evaluated once a sequencing project of the results from this study can be concluded since samples containing bacteria which is only originating from algae can be used for comparison. But it is also the case that we have only seen ocular and microscopic evidence of a mixture from a few of the possible colony types.

One can argue that no matter what might have contaminated the *E. pilosa* samples, as long as it was from the same oceanic surroundings as that species, the truly interesting thing is to set up a base camp of diversity in the *E. pilosa* natural environment, inside and outside the eukaryote. We can, however, only speculate on what might actually be happening in the life-cycle of *E. pilosa* and what kind of symbiotic or maybe parasitic encounters it may endure or be a part of.

### 1.2. *Alcyonium digitatum* origin

The results of the *A. digitatum* are, as with *E. pilosa*, no more than a hint of what might be. They are much more likely to be true symbionts of the eukaryote though, since the preparation used should preclude bacteria from the surface or surrounding of the organism. A sequencing project on this part is needed to shed more light on the situation.

## 2. Method evaluation

### 2.1. Cultivation of marine bacteria in non-marine surroundings

The method of cultivating marine bacteria in non-marine surroundings did work with some adjustments specific for this study. The biggest problem was the contamination of plates since there often was a need to put the plates in direct contact with laboratorial air.

This was of course done every time any colony type needed to be replated or needed to be checked more in detail.

A much better way to do this part of the project would be to have a laminate air flow working hood where all plates were incubated and handled. At least, a sterilized environment in a working hood would be desirable. The point is that it is so easy to contaminate these plates with slow growing bacteria with weeks of incubation that any other environment than the suggested (laminated air flow/sterile bench) will not work well. Until the sequencing of the results in this study can be made, there can be no certainty of what kind of colonies we have picked up in this study.

Also worth noting is that this study does not consider in any way the ultimate temperature and oxygen requirements of the bacteria. One has to remember that the native surroundings of the marine bacteria are 4 C and low oxygen levels. Therefore, the bacteria found may not be fully characteristic for the diversity in the organisms in their native environment. Still, this study can provide a few insights to that diversity.

### *2.1.1. Use of different plate media*

Interestingly, the M1 plates, containing the media supposed to be closest to the ocean, did not result in the heaviest bacterial growth. The greatest bacterial diversity was seen in the M6 plates which also contained the highest concentration of cycloheximide, ten times the concentration in the other media with this antibiotic. This is in a way very good, because it would seem that the bacteria not able to stand the high concentration of cycloheximide in M6 might be able to live on some of the other plates and therefore the colonies from this plate will be of great interest.

Another special observation from the plate media view is the M8 and M9 plates. These media had nothing in them except antibiotics and bacto agar. This was enough for them to show us a spectrum of 14 and 3 colony types respectively. Especially M8 is impressive with its 14 types. This is hopefully due to the low nutritional needs of marine bacteria and this plate will be of great interest in the sequencing project.

From studying where the growth has been favourable in the aspect of most variants of bacteria one can perhaps see the relation of the antibiotic rifampicin corresponding to little growth. In media M7 and M9, which both contain rifampicin, there are only 4 and 3 colony types respectively. This is so even though M7 is very similar in composition to M6 which should make M7 favourable to growth. This is not so and even though the nutrients are not very different and both contain peptone the overwhelming difference of 34 colony types between M6 and M7 could almost only be explained by the difference in antibiotics, i.e. rifampicin.

Finally one should consider the use of so many different kinds of media. As seen in the results this far, it has been positive to have as many as we did here since the goal of the project was to get a spectrum as wide as possible. This can only be achieved with respect to many different, but still alike, nutritional needs. Hopefully, this will result in a lot of different bacterial species in the continuation of this degree project.

The problem with the same view as above is that the work that is needed to maintain and process the information and live samples of bacteria is huge when

considering that everything you do one day will be easily multiplied by the next. This should not be forgotten as the results of such an experiment is based upon what care you can afford to give each part of the process. This is ultimately also an aspect of contamination issues which has been discussed earlier.

### *2.1.2. Fungal growth on media*

The fungal growth on the plates was a big problem. Mainly because when a plate was contaminated by fungus it would not be long until it was impossible to use in continued experiments. Probably, the fungus we encountered originated in the environment of the laboratory. Any way we could have altered the project without including a more secluded and sterile area would most probably have been of no difference in respect to the fungal contaminations.

Hopefully there is not too much information lost due to this problem, but that will remain unknown until more detailed investigations of *E. pilosa* and *A. digitatum* are carried out.

### 2.2. Extraction of bacterial DNA from eukaryotes

This simple method of getting at least some different strains of bacteria occupying the interior and exterior of a eukaryote in one single test would be most useful to scientists involved in any kind of diversity investigation. Currently we do not know if this assay worked or if it failed due to the same reason as before, there was no time for sequencing the PCR products.

Regardless, the primers used, which were the same as in the assay involving plated bacteria, would have a good chance of actually amplifying DNA from different bacterial species in a single tube. The problem with PCR is that any sequence fitting closely to the primers will yield enormous amounts of products. This is also the aim and the good side of PCR. Hence, the result would easily be dominated by one sequence.

To be able to identify more and different sequences one probably have to do a lot more pondering and tryout studies than we had time and possibilities to do. This is only a thought, though, since the result is not available until sequencing is done.

## 3. Where this study goes from here

A few looks into what has been said in this study will give any reader the view that more lab work needs to be done on this specific study and on the subject as a whole. Sequencing of the PCR products from the different bacterial colonies available here is the first thing, but additionally a thorough investigation should be done to determine what parts should be included in a new study. Hopefully this would verify what was concluded in this study.

The basic levels of sterility should be improved as well as a reduction of the width of medium types. At least if the study is not in the hands of more people to do the elaborative work. Since we have shown the diversity of bacteria that can be grown in the

lab, the study might be better by selecting a few plate types where we have seen the most diversified spectra occur.

## 4. Further studies worth looking for

Any study involving *E. pilosa* would be interesting to the future of this study since there is not very much work done on that species. The same can not entirely be said about *A. digitatum* since it is more widely used in studies than *E. pilosa*. However any study giving a hint on the lives of any of the two would simplify the search for diversity.

Also environmental studies involving deep sea species would potentially give more information as to whether any of the species could be involved in producing interesting substances or maybe even reducing toxic substances. Such a discovery would also give a much needed boost to this type of research.

Another kind of study worth looking for would be a study viewing new techniques involved in solving diversity problems. The ultimate test would be simply to be able to take a single test and run a PCR and get the diversity and species involved singularly from that. We are a few steps from that right now but it would not be a complete surprise if we in a few years could do just that.

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# Appendix

## 1. Media properties

All media are to be autoclaved for 15 min. in 121 degrees C and 1.1 Pa before thermally instable substances are added at approximately 55 degrees C and plates are poured.

| | | c (in media when stock solution used) | unit | amount | c (stock.) | solvent |
|---|---|---|---|---|---|---|
| **M1.** | Prior autoclave: | | | | | |
| | Marine agar | | (g) | 55,1 | | |
| | $H_2O$ | | (mL) | 1000 | | |
| **M3.** | Prior autoclave: | | | | | |
| | Actinomycete isolation agar | | (g) | 22 | | |
| | glycerol | | (g) | 5 | | |
| | $H_2O$ | | (mL) | 1000 | | |
| | After autoclave: | | | | | |
| | nalidixic acid | 10 µg/mL | (µL) | 500 | 20 mg/mL | $CH_2Cl_2$ |
| | cycloheximide | 10 µg/mL | (µL) | 1 000 | 10 mg/mL | EtOH 97% |
| | nystatin | 25 µg/mL | (µL) | 12 500 | 2 mg/mL | $H_2O$ |
| **M4.** | Prior autoclave: | | | | | |
| | Bacto agar | | (g) | 20 | | |
| | soluble starch | | (g) | 10 | | |
| | $K_2HPO_4$ | | (g) | 0,5 | | |
| | NaCl | | (g) | 20 | | |
| | filtered sea-$H_2O$ | | (mL) | 1 000 | | |
| | After autoclave: | | | | | |
| | nalidixic acid | 10 µg/mL | (µL) | 500 | 20 mg/mL | $CH_2Cl_2$ |
| | cycloheximide | 10 µg/mL | (µL) | 1 000 | 10 mg/mL | EtOH 97% |
| | nystatin | 25 µg/mL | (µL) | 12 500 | 2 mg/mL | $H_2O$ |
| **M5.** | Prior autoclave: | | | | | |
| | Bacto agar | | (g) | 20 | | |
| | yeast extract | | (g) | 4 | | |
| | malt extract | | (g) | 10 | | |
| | dextrose | | (g) | 4 | | |
| | NaCl | | (g) | 20 | | |
| | filtered sea-$H_2O$ | | (mL) | 1 000 | | |
| | After autoclave: | | | | | |
| | nalidixic acid | 10 µg/mL | (µL) | 500 | 20 mg/mL | $CH_2Cl_2$ |
| | cycloheximide | 10 µg/mL | (µL) | 1 000 | 10 mg/mL | EtOH 97% |
| | nystatin | 25 µg/mL | (µL) | 12 500 | 2 mg/mL | $H_2O$ |

**M6.**

| Prior autoclave: | | | | | |
|---|---|---|---|---|---|
| Bacto agar | | (g) | 20 | | |
| soluble starch | | (g) | 10 | | |
| yeast extract | | (g) | 4 | | |
| filtered sea-$H_2O$ | | (mL) | 1 000 | | |
| After autoclave: | | | | | |
| peptone | 2 mg/mL | (mL) | 20 | 100 mg/mL | $H_2O$ |
| cycloheximide | 100 µg/mL | (µL) | 10 000 | 10 mg/mL | EtOH 97% |

**M7.**

| Prior autoclave: | | | | | |
|---|---|---|---|---|---|
| Bacto agar | | (g) | 20 | | |
| mannitol | | (mg) | 500 | | |
| filtered sea-$H_2O$ | | (mL) | 1 000 | | |
| After autoclave: | | | | | |
| peptone | 0.1 mg/mL | (µL) | 1 000 | 100 mg/mL | $H_2O$ |
| rifampicin | 5 µg/mL | (µL) | 2 000 | 2.5 mg/mL | $H_2O$ |
| cycloheximide | 10 µg/mL | (µL) | 1 000 | 10 mg/mL | EtOH 97% |

**M8.**

| Prior autoclave: | | | | | |
|---|---|---|---|---|---|
| Bacto agar | | (g) | 20 | | |
| filtered sea-$H_2O$ | | (mL) | 1 000 | | |
| After autoclave: | | | | | |
| novobiocin | 25 µg/mL | (µL) | 2 000 | 12.5 mg/mL | $H_2O$ |
| cycloheximide | 10 µg/mL | (µL) | 1 000 | 10 mg/mL | EtOH 97% |

**M9.**

| Prior autoclave: | | | | | |
|---|---|---|---|---|---|
| Bacto agar | | (g) | 20 | | |
| filtered sea-$H_2O$ | | (mL) | 1 000 | | |
| After autoclave: | | | | | |
| rifampicin | 5 µg/mL | (µL) | 2 000 | 2.5 mg/mL | $H_2O$ |
| cycloheximide | 10 µg/mL | (µL) | 1 000 | 10 mg/mL | EtOH 97% |