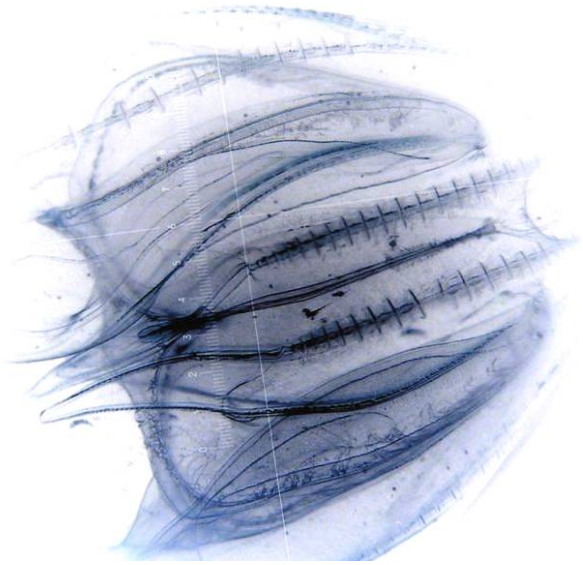
Invasion Biology of *Mnemiopsis leidyi* and its symbionts



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Vorgelegt von

Sven Hammann

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Erster Gutachter:	PD Dr. rer. nat. Martin Zimmer
Zweiter Gutachter:	Prof. Dr. rer. nat. Ulrich Sommer
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Für alle, die an mich geglaubt haben.

Danke!

Abstract

Symbiotic interactions are one of the most important biotic factors for every living organism, and they have stimulated growing scientific interest during the last few years. Symbiosis is understood here as the umbrella term for all forms of coexistence of individuals of different species, be they parasitic, mutualistic or commensalistic. For an invasive organism it has to be considered that not only itself has to face the new environmental conditions but also its symbionts. Recent studies have shown that changing environmental conditions can change the kind of relationship between a host and its symbionts in fundamental ways. Mutualists can become parasites and vice versa.

The comb jelly *Mnemiopsis leidyi* (Ctenophora), also known as sea walnut, has found to be a strong invader and a strong competitor to locally established organisms - especially fishes and planktonic organisms - for about three decades now. After the invasion of the Black Sea in the 1980s the sea walnut spread into the neighboring seas in the following years. In October 2006 it was first reported in the Baltic Sea, in the North Sea it was found a few months later. An almost continuous monitoring of *M. leidyi* in the Baltic Sea since its first occurrence opened the possibility to learn more about the underlying mechanisms which determine whether an invasion proves successful or not.

In this thesis, a one-year monitoring (2008) of *M. leidyi* is presented and discussed. The distribution and abundances of the sea walnut indicate a stable (at least for the moment) population in the western Baltic Sea which reaches up to Fehmarn and the bight of Lübeck.

Furthermore, *M. leidyi* was screened for bacterial and archaeal symbionts; "higher" symbionts - like amoeba or anemone - had not been found so far in the Baltic Sea. The 16sRNA gene-based screening of whole-body extracts on the one hand gave hints for a stable and exclusive symbiotic community. On the other hand, the surface epithelium - as the area of the very first contact to a (novel) environment and potential symbionts - seems to be completely void of bacteria. The underlying mechanisms, potential benefits and consequences of this finding are totally unclear up to now. To gain insight into this astonishing discovery, several methods for fixation of the animals without preparation artifacts were tested. The prepared surfaces should allow for structure analysis through light- and electron microscopy.

Finally, it is discussed to use *M. leidyi* - and invasive species in general - as a "space-for-time" approach to study the effects of long term climate and environmental changes on species and their symbionts under natural conditions.

Zusammenfassung

Symbiotische Wechselbeziehungen gehören zu den wichtigsten Faktoren im Leben eines jeden Organismus. Hierbei ist Symbiose als Überbegriff für jegliche Form des Zusammenlebens zweier Individuen verschiedener Artzugehörigkeit zu verstehen, egal ob parasitisch, mutualistisch oder kommensalistisch.Eine invasive Art setzt sich nicht alleine für sich mit neuen Umweltbedingungen auseinander, sondern immer im Verbund mit ihren Symbionten. So konnte gezeigt werden, dass sich die symbiotischen Beziehungen radikal verändern können wenn sich die Umweltbedingungen ändern: Mutualisten werden zu Parasiten und umgekehrt.

Die Rippenqualle *Mnemiopsis leidyi* (Ctenophora), umgangssprachlich als Meerwalnuss bezeichnet, ist seit drei Jahrzehnten als eine hochinvasive Art und starker Konkurrent für etablierte Spezies - speziell Fische, aber auch andere planktische Organismen - bekannt. Nach der Einschleppung ins Schwarze Meer in den 1980er Jahren verbreitete sie sich unaufhaltsam in die benachbarten Meere. Im Oktober 2006 wurde sie zuerst in der Ostsse und kurz darauf auch in der Nordsee gesichtet. Ein seither nahezu kontinuierliches Monitoring eröffnet die Möglichkeit, mehr über Mechanismen zu lernen die entscheiden ob eine Invasion erfolgreich verläuft oder nicht.

In der vorliegenden Arbeit wird zunächst ein einjähriges Monitoring (2008) von *M.leidyi* vorgestellt und diskutiert. Die beobachteten Verteilungsmuster und Abundanzen legen eine stabile (zumindest für den Moment) Population in der westlichen Ostsee nahe, die im Osten bis Fehmarn und zur Lübecker Bucht reicht.

Weiterhin wurde *M. leidyi* auf bakterielle Symbionten untersucht; „höhere“ Symbionten wie Amöben oder Anemonen konnten für die Ostsee bisher nicht nachgewiesen werden. Das 16sRNA Gen-basierte Screening ergab eine von der Umgebung distinkte und stabile Lebensgemeinschaft. Andererseits erwies sich die Oberfläche - als Zone des ersten Kontakts mit einer neuen Umwelt und potentiellen Symbionten - als völlig bakterienfrei. Die zugrundeliegenden Mechanismen sowie daraus resultierende Vor- und Nachteile sind bisher völlig unbekannt. Um diese erstaunliche Entdeckung besser zu verstehen wurden verschiedene Methoden zur Fixierung der Tiere erprobt. Diese sollen eine Betrachtung des ungestörten und unzerstörten Epithels mittels Licht- und Elektronenmikroskopie ermöglichen.

Abschließend wird diskutiert, *M. leidyi* - und invasive Arten im Allgemeinen - als Modellorganismen für einen „Ort-statt-Zeit“ Ansatz zu nutzen, um Langzeiteffekte von Umwelt- und Klimaveränderungen auf Arten und ihre Symbionten zu untersuchen.

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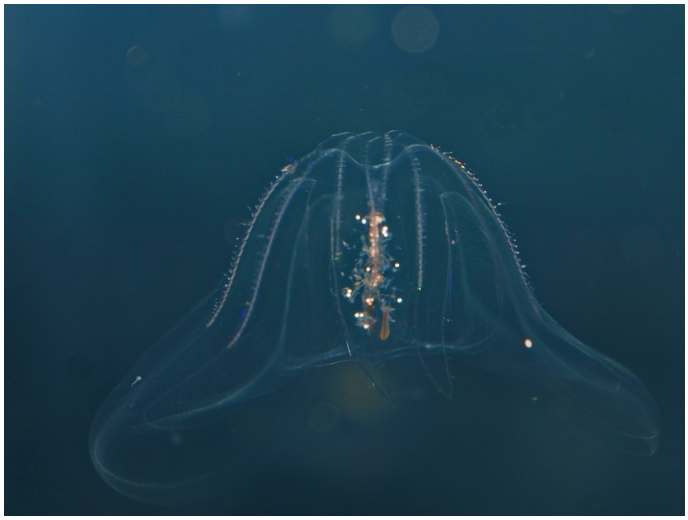
1. GENERAL INTRODUCTION



"Ctenophores are somehow an alternative way of being a jelly."

(Richard Dawkins 2004)

For approximately three decades now, the comb jelly *Mnemiopsis leidyi* (Ctenophora) Agassiz 1865 is known to be an extremely successful invader. During this time span it spread across nearly all European coastal waters. From its native environment - the coastal and estuarine waters of the east coasts of North and South America (Kremer, 1994) - it was introduced into the Black Sea in the beginning of the 1980s (GESAMP, 1997; Shiganova, 1998; Shiganova et al., 2003), likely accidentally by ship ballast water. In the following years, the sea walnut spread through the neighboring waters, particularly the Sea of Azov (Shiganova, 2000), the Caspian Sea (Ivanov et al., 2000), the Aegean Sea (Kideys and Niermann, 1994) and the Mediterranean Sea (Isinibilir and Tarkan, 2002; Fuentes et al., 2009); for a detailed chronological history see also the report of GESAMP (1997). The invasion of the Baltic and the North Sea seemed to be only a matter of time and indeed the occurrence of the comb jelly was reported in both areas during the last years; 2006 in the Baltic (Javidpour, 2006) and shortly later in the North Sea (Faasse and Bayha, 2006; Boersma et al., 2007). In contrast to a prediction, which proposed an invasion would come from the southern European seas via the various connecting water streets, recent population genetic studies proved several independent invasion events out of the native habitat for the Mediterranean, the Baltic, and the Northern Sea (Reusch et al., 2010); a model about invasion pathways and dispersal of *Mnemiopsis leidyi* through the Baltic Sea can be found in Lehmann and Javidpour (2010).

Taking possession of such different areas like the Baltic, the Black Sea, or the Mediterranean implies a high potential to adapt to, or acclimate with, different environmental conditions. As a matter of fact *Mnemiopsis leidyi* is highly tolerant to temperature (-2 °C up to 32 °C) and to salinity (2 psu up to 38 psu) (Purcell et al., 2001). Besides its tolerance to environmental conditions, the sea walnut has a voracious feeding habit, a high reproduction rate and a high potential for regeneration (Deason, 1982; Mills, 1995; Shiganova, 1998; Henry and Martindale, 2000; Javidpour et al., 2008; Pang and Martindale, 2008) which render it to be a superior competitor to native species in the invaded areas.

The classification of the Ctenophora is still under discussion. Different studies based on 18S rRNA genes (Podar et al., 2001) supported that the traditional system needs revision, but as the case stands this is still an ongoing progress; among other reasons because of sparse morphological and especially molecular data. The following system is based on the information collected and published by Mills (1998) and is giving a short overview of the known classes, orders, families and the number of genera. Because of the morphological plasticity of the whole phylum there are a lot of known - and still some unknown - synonyms and redundancies; Mills suggests that there are 100 to 150 known “good species” known so far.

- Phylum: Ctenophora, Eschscholtz 1829
  - Class: Tentaculata, Eschscholtz 1825
    - Order: Cydippida, Gegenbaur 1856
      - Family: Haeckeliidae, Krumbach 1925 (2 genera)
      - Family: Ctenellidae, Carré & Carré 1993 (1 genus)
      - Family: Bathyctenidae, Mortensen 1932 (1 genus)
      - Family: Lampeidae, Krumbach 1925 (1 genus)
      - Family: Pleurobrachiidae, Chun 1880 (7 genera)
      - Family: Euplokamidae, Mills 1987 (1 genus)
      - Family: Cryptocodidae, Leloup 1938 (1 genus)
      - Family: Mertensiidae, Agassiz 1860 (3 genera)
      - Family: Dryodoridae, Harbison 1996 (1 genus)
    - Order: Platyctenida, Bourne 1900
      - Family: Ctenoplanidae, Willey 1896 (1 genus)
      - Family: Tjalfiellidae, Komai 1922 (1 genus)
      - Family: Lyroctenidae, Komai 1942 (1 genus)
      - Family: Savangiidae, Harbison & Madin 1982 (1 genus)
      - Family: Coeloplanidae, Willey 1986 (2 genera)
  - Class: Cyclocoela, Ospovat 1985
    - Order: Cambojiida, Ospovat 1985
      - Family: Cambojiidae, Ospovat 1985 (1 genus)
    - Order: Ganeshida, Moser 1908
      - Family: Ganeshidae, Moser 1907 (1 genus)
    - Order: Cryptolobiferida, Ospovat 1985
      - Family: Cryptolobatidae, Ospovat 1985 (2 genera)
    - Order: Thalassocalycida, Madin & Harbison 1978
      - Family: Thalassocalycidae, Madin & Harbison 1978 (1 genus)
    - Order: Lobata, Eschscholtz 1825
      - Family: Bathocyroidae, Harbison & Madin, 1982 (1 genus)
      - Family: Bolinopsidae, Bigelow 1912 (3 genera)
      - Family: Leucotheidae, (Lesson 1843) (1 genus)
      - Family: Ocyropsidae, (Lesson 1843) (2 genera)
      - Family: Eurhamphaeidae, Agassiz 1860 (3 genera)
      - Family: Lampoctenidae, Harbison, Matsumoto & Robison 2001 (1 genus)
      - Family: Lobatolampeidae, Horita 2000 (1 genus)
    - Order: Cestida, Gegenbaur 1856
      - Family: Cestidae, Gegenbaur 1856 (2 genera)
  - Class: Nuda, Chun 1879
    - Order: Beroida, Eschscholtz 1825
      - Family: Beroidae, Eschscholtz 1825 (2 genera)

Following the system above, *Mnemiopsis leidyi* is located in the family Bolinopsidae Bigelow 1912, within the order Lobata Eschscholtz 1825, within the class Cyclocoela Ospovat 1985. The genus *Mnemiopsis* currently contains two species; *Mnemiopsis leidyi* and *Mnemiopsis gardeni* L. Agassiz 1860. But even here the correct nomenclature is under discussion, maybe *M. gardeni* is also synonymous to *Mnemiopsis leidyi*. On the other hand it is questionable, wether *Mnemiopsis mccradii* is really a synonym for *M. leidyi*. The other two genera of the Bolinopsidae are called *Bollinopsis* Agassiz 1860, and *Lesueuria* Milne & Edwards 1841, with the first containing eight and the latter four species.

Although at first sight the Ctenophora might look like the commonly known jellyfish (Cnidaria), these two taxa are not closely related. Historically, the two taxa have been placed together as sister groups in the taxon Coelenterata, close to the base of the Metazoan Tree of Life. Nowadays it is widely accepted that they are not sister taxa to each other, and Coelentarata is a polyphyletic group; but an exact position of the comb jellys is still not established finally. As a matter of fact, the exact relationship of Cnidaria, Bilateria, and Ctenophores is still not clear and three possibilities are discussed. Today most authors place the Ctenophora as a sister group to the Cnidaria and Bilateria, called the Planulozoa hypothesis (Wallberg et al., 2004; Medina et al., 2001). One reason for this, not being considered before, might be the difficulty to accept seemingly higher developed animals being more distantly related to the Bilateria than seemingly lesser evolved ones (the Ctenophores). For a summary of arguments of all possible relationships see Minelli (2009, p.46ff). He suggests evolutionary development as a potential field of research to solve the problems in ctenophore's phylogenetics (Minelli, 2007; Minelli et al., 2006). One of the latest contributions to this topic places the Ctenophora at the very base of the Metazoan Tree of Life, which might mean that they are even older than the sponges, which are the presumably oldest line of multicellular animals on earth (see Hejnol et al. (2009) and Dunn et al. (2008)). This would implicate several questions and conclusions such as: Have features found in Ctenophores and Bilateria evolved several times? Did the sponges loose some features, which common ancestors have had? Is there one root of bilaterian symmetry or are there several?

Some of the major differences between Ctenophora and Cnidaria are the type of locomotion (ciliar vs. muscular), the ultrastructure of the sperm cells (acrosome vs. acrosomal lamella), and the absence of own stinging cells (nematocytes) in Ctenophora. Ctenophora have developed a totally different type of "catching-prey-cells", which are a kind of "wrapping"-cells. However, some species are able to use additionally the nematocytes of their prey out of the group of the Cnidaria. These cells are then called Kleptocnides, but are never a product of the Ctenophore itself. Finally, Cnidaria tend to have complex developmental cycles with altering stages of

free-living and sessile animals. In contrast, all known species of Ctenophora have a direct development from the egg to the adult animal. *Mnemiopsis leidyi* has - like other Ctenophores - a high regeneration ability (Henry and Martindale, 2000) and a high reproduction rate (Javidpour et al., 2008; Deason, 1982). They can start to reproduce in larval stage (dissogony) and continue, after a short break during growing up, until they die (Martindale, 1987). In this way, they are able to build up high abundances in a quite short time if environmental conditions fit their needs. In the Kiel Bight it was observed that population sizes got 10 times higher from one week to another (Javidpour et al., 2008; Schroedter, 2008). As most Ctenophores, *Mnemiopsis leidyi* is a self-fertilizing hermaphrodite. The male and female gonads are placed alternating and close to the combs in the inner tissue layer (Baker and Reeve, 1974). Eggs and sperms are released into the water via gonopores. After fertilization, the embryo develops rapidly inside the egg, and a free-living larva is hatching after 24 hours. The larva is a cydippid-larva, which looks like a small version of the adult animal but bearing two tentacles. These get lost during further development, like in most groups of the Ctenophora with exception of the class Tentaculata Eschscholtz 1825, which keep their tentacles all life long. The sea walnut is a voracious predator on zooplankton but does not spurn phytoplankton at all (Sullivan and Gifford, 2007). Observations under high prey densities have shown that, when their gastrovascular system is filled with copepods, they discard their stomach content and start feeding again; reasons for this kind of feeding habit remain unclear.

As mentioned before, *Mnemiopsis leidyi* is known as a potential and powerful invader for about 30 years now. The breakdown of the fish stocks and therefore the fishing industries around the Black Sea in the beginning of the 1980s is usually correlated to the occurrence of the sea walnut in these waters (Shiganova, 2000). Today the comb jelly is in parts rehabilitated (Gucu, 2002; Bilio and Niermann, 2004; Kideys, 2002). It is widely accepted that *Mnemiopsis leidyi* has established after the fish stocks were massively overfished and then - as a competitor and potential predator on fish eggs and larvae - accelerated the decline in fish abundances. Nevertheless, with its remarkable tolerance against abiotic factors like salinity and temperature, its high reproduction and regeneration potentials and its omnivoric voracious feeding habits, the sea walnut is a potential - and as a competitor and predator potential harmful - invader in the Baltic and the North Sea. Since the first report in 2006 in the Baltic (Javidpour, 2006), the comb jelly is monitored continuously at least in the Kiel Bight. The abundances during the blooms were high every year. Interestingly in 2011 not a single *Mnemiopsis* was found in the Kiel Bight. The reasons for this remain unknown until today, but discussed explanations are high numbers of the Cnidarian genera of *Aurelia* and *Cyanea* during spring and

summer, disturbed water influxes from the North Sea, the long and cold winter in 2010/11 and/or other changes in abiotic factors; the future will show whether *Mnemiopsis leidyi* is still a member of the Baltic Sea plankton community or - if not - if it will reestablish again.

The last paragraphs just showed in short summary that *Mnemiopsis leidyi* has a couple of features to be considered as a strong invader; namely high reproduction rate, high regeneration ability, and an omnivorous feeding habit. In an investigation of invasive species and invasive potential of alien species, one point is usually not under discussion or even observation. It is totally unclear whether or not the symbionts of an invasive species play a role during the process of invading new areas. Especially the symbionts on the inner and outer surfaces are of interest in this context because the surfaces of an organism are always the areas of first contact with a new environment and its inhabiting organisms. How do symbiotic interactions change when the environmental conditions change? How is the symbiotic community affected by new potential symbionts? Are symbionts a drawback or a driver for invasive success? And looking forward, studying questions like these can give insight in questions of global change, too. How do organisms face a changing environment? Do symbiotic interactions support or hinder adapting processes? As a second point, the study of an invasive species and its symbionts should be seen as a "space-for-time" approach to study future scenarios in the light of global change.

Maybe at this point a short specification about the word "symbiosis" is appropriate. In this thesis the word is used in its original meaning defined by Anton de Bary in 1878 at the 51st meeting of German natural and medical scientists in Kassel: "Symbiosis is the living together of two individuals of different species." This includes explicitly parasitism, commensalism and mutualism. Especially in the German language area symbiosis is often used synonymous to mutualism.

So the overall topic of this thesis is not *Mnemiopsis leidyi* alone but *Mnemiopsis leidyi* AND its symbionts, that is to say the holobiont *M. leidyi* (compare Margulis (1993) and Zilber-Rosenberg and Rosenberg (2008)).

Still, the first part deals with a one-year-monitoring of the western Baltic Sea (2008) in four different regions, namely Kiel Bight, Eckernförde Bight, Flensburg Bight and Fehmarn. In each of these regions four to five stations were sampled once a month in three replicates. Several abiotic (temperature, depth, salinity) and biotic (concentrations of phyto- and zooplankton, content of chlorophyll a, number of fish larvae, plankton composition) factors were measured. Beside its sheer abundances, the maturity, size, and size composition of the populations of *Mnemiopsis leidyi* was recorded. During this process a number of individuals of *M. leidyi* were observed microscopically for symbionts and others were frozen for later laboratory studies on bacterial and archaeal symbionts.

The second part focuses on the laboratory work, which includes the development of adequate methods for DNA extraction as well as the results. Two main points can be derived from this part. On the one hand, there are stable communities of bacteria associated with the sea walnut, independently from which sampling point in the western Baltic the host animals originate from. On the other hand, even more fascinating, the surface itself proved to be totally void of bacteria. This means that *Mnemiopsis leidyi* somehow keeps its surface sterile. If this is supported in further studies, we will have here the first report of a completely sterile surface on a living animal in the ocean.

Finally, the third part will focus on the development of methods to visualize the surface of the comb jelly and its structures. On the one hand to give optical support for the molecular findings presented in part 2, and on the other hand to understand the underlying mechanisms for this finding. The existing methods for (electron-)microscopy include rather harsh conditions, which prevent us from studying the comb jelly's surface in a natural, undestroyed state. Unfortunately, in 2011 not a single individual of *M. leidyi* was found in the Baltic Sea. So these studies could not be completed before the submission of this thesis.

The very last chapter is a concluding discussion of all presented studies, followed by a short outlook on future work worth to be done.

2. PART 1 - MONITORING

"At first view Ctenophores are little more than organized seawater."

(unknown)

2.1 Abstract

During the year 2008, the distribution of the invasive sea walnut *Mnemiopsis leidyi* (Ctenophora) in the south western Baltic Sea was monitored to shed light on its settlement and distribution since its first observation in 2006. Four different regions (Kiel Bight, Eckernförde Bight, Flensburg Bight, coast of Fehmarn) were sampled monthly for the occurrence and abundances of comb jellies and cnidarian jelly fish. Water samples were analyzed for temperature, salinity, fish eggs and -larvae, as well as phyto- and zooplankton compositions and abundances. The Bight of Lübeck was sampled only twice, in April and October, because of weather and ship-time limitations.

The data clearly show the main bloom of *M. leidyi* occurring in autumn, around September, in contrast to the two yearly blooms in spring and autumn in its native habitat, the eastern coasts of North and South America. Additionally, it seems that the edge of distribution of the comb jelly is somewhere between Fehmarn and the Lübeck Bight, at least for the moment. Furthermore, the findings suggest that *Mnemiopsis leidyi* does not affect the fish stocks in the western Baltic Sea directly, although nothing can be said so far about indirect long term effects.

2.2 Introduction

Since about three decades now, the sea walnut *Mnemiopsis leidyi* (Ctenophora) is known to be a successful and potentially harmful invader. Its invasion of the Black Sea started during the 1980's (Shiganova, 1998; GESAMP, 1997). In the upcoming years it spread into the neighboring waters, i.e. the Sea of Azov (Shiganova, 2000), the Caspian Sea (Ivanov et al., 2000), and the Mediterranean Sea. Its expansion in the latter is still an ongoing process and it reached Spanish waters by coming from the east just recently (Fuentes et al., 2009).

After its arrival in the Black Sea, the fish stocks - and, related to it, the fishery - collapsed. A long time *M. leidyi* was assumed to be exclusively responsible for this decline, though it is in parts rehabilitated today. It was shown that the fish populations had been totally exhausted by overfishing before the comb jelly arrived (Gucu, 2002). Nevertheless, with its voracious feeding habits, its high reproduction rate, its enormous regeneration potential and its remarkable tolerance towards environmental conditions like temperature and salinity, *Mnemiopsis leidyi* is a strong competitor to all native species, especially when the latter are already at their ecological limit.

The native habitats of the sea walnut are the coasts and estuaries of the North and South American east coasts. Within its native area *M. leidyi* tolerates a wide range of abiotic conditions, for example conditions of temperature (-2 °C up to 32 °C) and salinity (from 2 psu up to 38 psu) (Purcell et al., 2001; Kremer, 1994; Sullivan et al., 2001). Of course *M. leidyi* has to cope with a high diversity of potential prey, predators and symbionts, too.

The most recent invasive event known so far took place in 2006 in the Baltic Sea (Javidpour, 2006; Hansson, 2006) and the North Sea (Boersma et al., 2007). Of special interest is the invasion of the Baltic Sea. On the one hand, the Baltic Sea is a quite young water body, less than 10.000 years old. On the other hand, being the biggest sea of brackish water in the world, it is isolated from the oceans and connected only through small water streets to the North Sea. Thus, it has a wide range of salinity from 20 psu in the belt area down to 1 psu in the north eastern parts. These obstacles, a relatively young ecosystem and harsh and varying conditions, caused a (relatively) poor species community. Nevertheless an established community exists and the question will be how the invader will interact with the native organisms. A special focus is set here to the moon jellyfish *Aurelia aurita* (Cnidaria), which is potentially a strong competitor to *M. leidyi* for the same ecological niche. It is totally unclear what will happen: Will one species drive out the other? Will *Mnemiopsis leidyi* harm the fish stocks and therefore the fish industries of the Baltic Sea? Or will the sea walnut adapt without profound implications to

the dynamic system of the Baltic Sea?

A last but nonetheless important point is that the Baltic Sea is an extremely "used" waterbody - in terms of fishery, tourism, seafaring - and in comparison a well-studied and regularly observed one, too. Hence, it is very likely that *Mnemiopsis leidyi* was detected quite early after its appearance. This provides a unique possibility to study an invasive process from the very beginning.

2.3 Material & Methods

Data was collected from February to November 2008 during trips with the research vessel "FK Littorina" of the IFM - GEOMAR, Kiel. The sampling in December failed because of weather conditions. Overall, five locations (Figure 2.1) were sampled with four to five stations each. The stations were sampled monthly, except for the Bight of Lübeck (stations 20 through 23), which was sampled only in April and October 2008 due to weather and ship-time limitations.

The coordinates of the Lübeck Bight stations (see table 2.1) had to be restored with the help of the original cruise map (see figure 2.1) and Google earth, version 6.1.0.5001, because the data-log-file of the research vessel was lost.

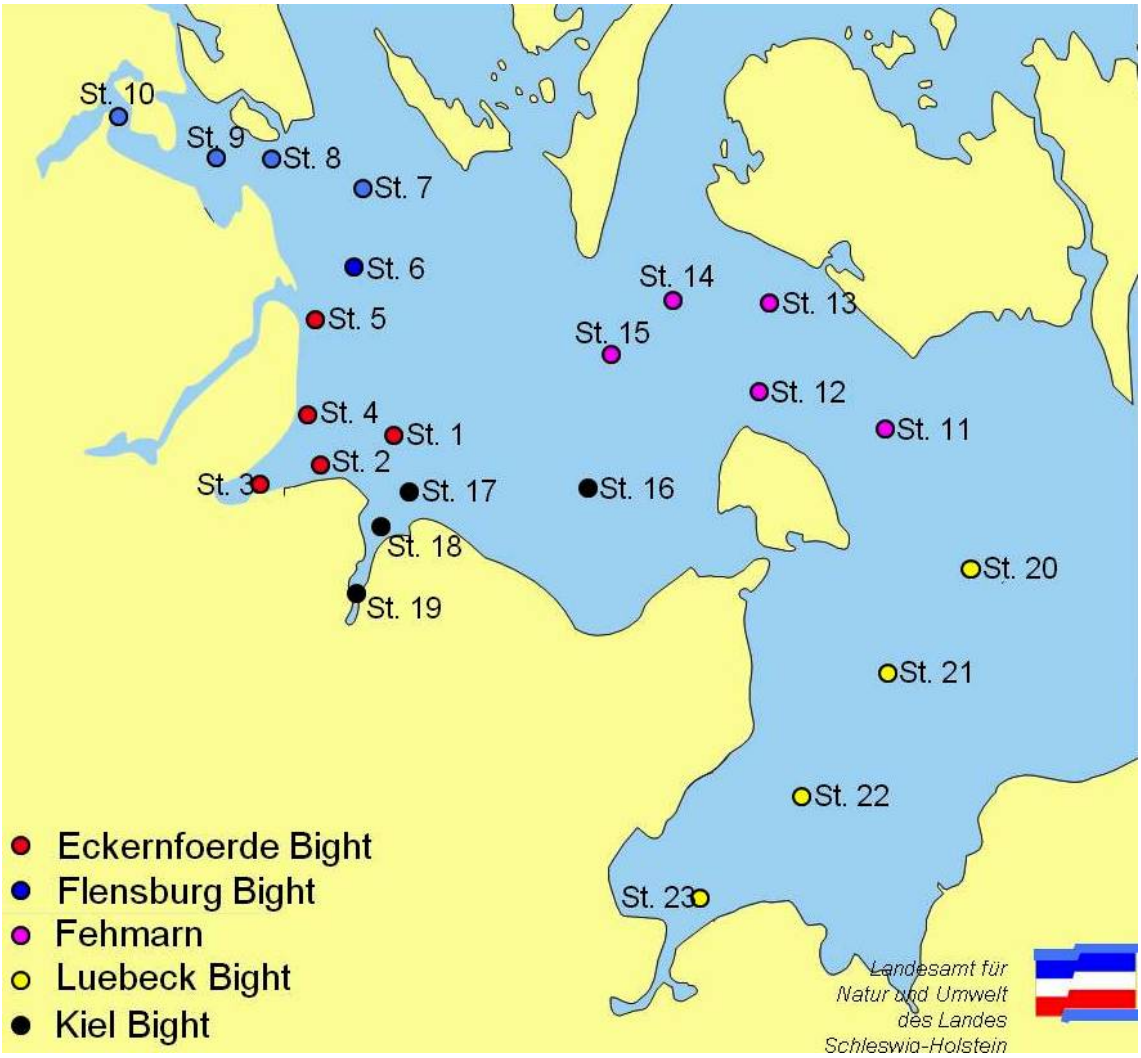


Fig. 2.1: Stations sampled in the south western Baltic Sea during the monitoring of *M. leidyi*, 2008.

Tab. 2.1: Coordinates and station numbers of the sampling stations during the *M. leidyi* monitoring in 2008. Additionally the stations are sorted from west to east and named in the text as x'.

Area	Stations	Coordinates	Stations from west to east
Eckernförde Bight	1	54°29.40N 010°13.00E	12'
	2	54°30.30N 010°06.00E	7'
	3	54°29.00N 009°58.00E	3'
	4	54°31.20N 010°02.00E	5'
	5	54°37.50N 010°08.60E	6'
Flensburg Bight	6	54°50.70N 009°38.60E	8'
	7	54°50.00N 009°38.60E	9'
	8	54°49.60N 009°58.60E	4'
	9	54°47.30N 010°00.00E	2'
	10	54°40.70N 010°09.80E	1'
Fehmarn	11	54°37.02N 010°41.94E	19'
	12	54°36.99N 010°52.99E	17'
	13	54°36.99N 011°07.95E	18'
	14	54°33.99N 011°10.96E	16'
	15	54°31.27N 011°19.69E	15'
Kiel Bight	16	54°30.99N 010°40.91E	14'
	17	54°28.36N 010°18.20E	13'
	18	54°24.36N 010°12.02E	11'
	19	54°21.64N 010°10.35E	10'
Lübeck Bight	20	54°22.58N 011°32.41E	23'
	21	54°17.21N 011°23.35E	22'
	22	54°08.27N 011°10.02E	21'
	23	54°01.39N 010°59.24E	20'

All samples were taken in three replicates. Live counts were done on catches made with a WP3-net (mesh size: 500 µm), all samples were directly counted on board (Cnidaria, Ctenophora, fish eggs and larvae). The individual size - umbrella diameter for Cnidaria and oral-aboral lengths for the Ctenophora - was also recorded. Samples for copepod counting were taken with a WP2-net (mesh size: 200 µm) and conserved in 5%-formol-solution. Also stored in 5%-formol-solution were samples taken with a plankton net (mesh size: 100 µm). Water samples (each 0.5 L) were taken at three depths (bottom, surface, column; depths varied between 10 and 30 meters) at every station and mixed thoroughly. Out of this, 0.5 L were filtered (Wattman, 0.2 µm, glass fiber) and frozen at −20°C for later chlorophyll a (Chla [µg /L])-measurements. Extraction and calculation for Chla was done according to Jeffrey and Humphrey (1975): Chla [µg /L] = (11.85*D665-1.54*D647-0.08*D630)*v/(L*V), with Dxxx = corrected absorption at wavelength xxx, v = volume of acetone, V = volume of filtered water sample, L = length of the cuvette. For nutrient determinations (sodium, ammonium, ortho-phosphate, silicate) three scintillation vials were filled with 20 mL filtered seawater and frozen at −20°C. Analysis was done with a C/N-Analyzer (FLASH 2000, Organic Elemental Analyser, Thermo Scientific) and an Autoanalyser (SAN plus System, Skalar). To determine ciliates and phytoplankton, about 300 mL of mixed seawater were fixed and stained in 1% Lugol's-solution. These samples were stored in brown-glass bottles and counted under a standard microscope in the laboratory. Unless otherwise indicated all chemicals were purchased from Merck KGaA.

Statistical analyzes and graphs were done with R (RCoreTeam, 2012) and Sigma Plot 10.0 (Systat Software, San Jose, CA).

2.4 Results

Three out of four sampling sites showed similar population patterns during the sampling year 2008: the bight of Eckernförde (figure 2.2), the bight of Flensburg (figure 2.3), and the coast of Fehmarn (figure 2.4). A first bloom of *A. aurita* appeared in February/March/April, followed by a peak in the copepoda abundances in the following month. Both numbers decreased during the summer months. *M. leidyi* was not found during this time but around September/October the comb jelly abundances exhibited very high numbers - up to 70 individuals per cubic meter. In Eckernförde and Fehmarn *A. aurita* and the copepods are showing a second but much smaller bloom just before the increase of the *M. leiyi* population. In the bight of Kiel (figure 2.5) the situation presents itself slightly different. The "spring-peaks" of *A. aurita* and of the copepods start a little later (April to May), and although the summer decrease is clearly visible, the abundances stay high in June/July. The second bloom of these two species precedes the *M. leidyi*-peak in September/October.

In figures 2.6 and 2.7, the all-year abundances of *M. leidyi* and *A. aurita* are shown from west (station 1') to east (station 23') as absolute and log-normalized quantities. Here all replicates and months are pooled together; note that the station numbering x' does not correspond to the numbers in figure 2.1, a key is given in table 2.1. Additionally, the numbers according to figure 2.1 are given in parenthesis in the text. On first view the abundances are equally high in the observed areas. In the bight of Lübeck (stations 20' to 23') the abundances tend to be a little bit smaller than in the other areas. The most inner stations of the bights (3' (3), 10' (19) and 20' (23)) show the lowest abundances with the exception of station 1' (10), the inner station of the bight of Flensburg. Figure 2.7 shows that *Mnemiopsis leidyi* , *A. aurita*, jellyfish in general (jelly richness), and the copepods as well occur in the same areas.

The negative correlation (Pearson coefficient: -0.24) in the occurrences of the jellyfish *A. aurita* and the comb jelly *M. leidyi* using the bight of Eckernförde as example is shown in figure 2.8. The other areas would show similar correlations, as the two species are rarely found simultaneously. During spring and summer the moon jellyfish dominates, whereas in autumn the sea walnut is the dominating species in the macrozooplankton (see also figures 2.2 to 2.5).

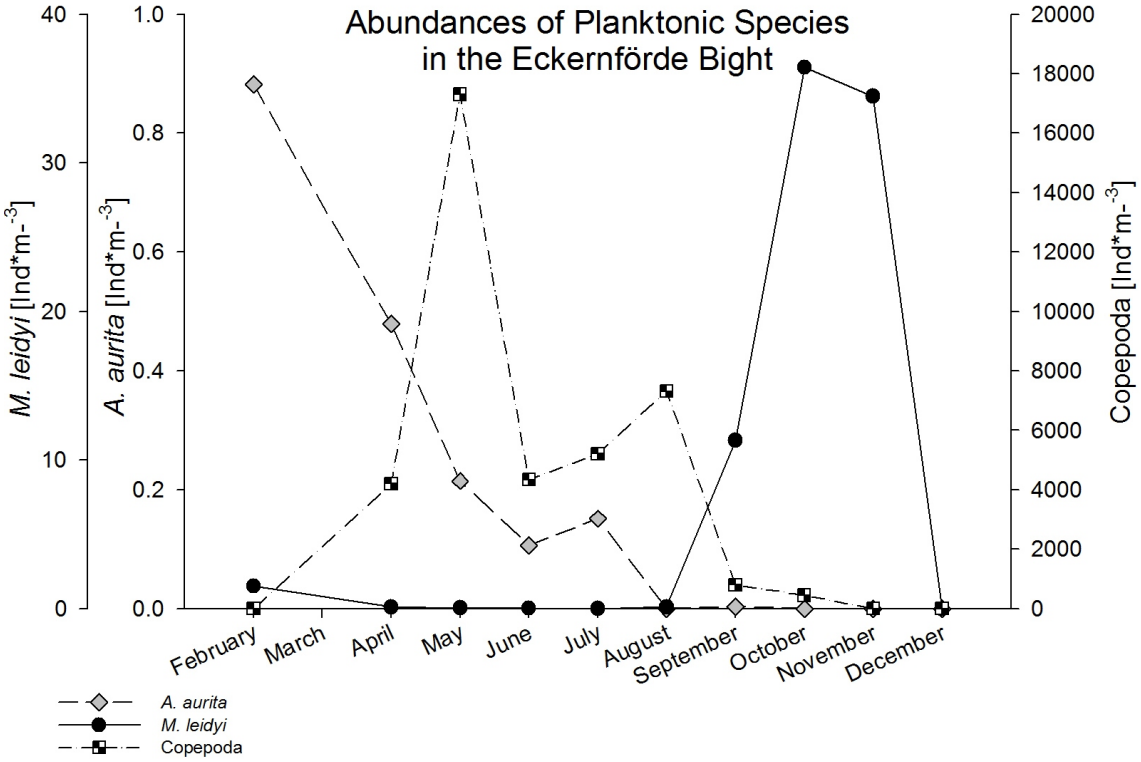


Fig. 2.2: Abundances of *M. leidyi*, *A. aurita*, and copepoda in the bight of Eckernförde in the year 2008. Five sampling locations each with 3 replicates are pooled for every month.

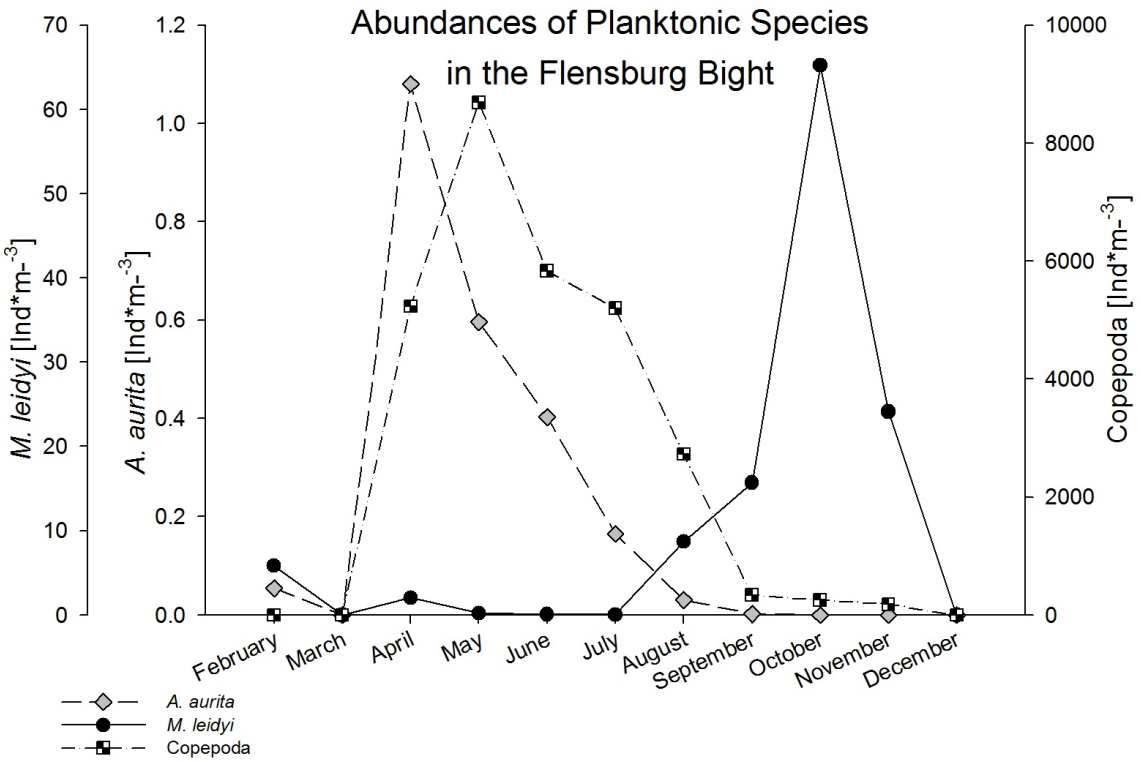


Fig. 2.3: Abundances of *M. leidyi*, *A. aurita*, and copepoda in the bight of Flensburg in the year 2008. Five sampling locations each with 3 replicates are pooled for every month.

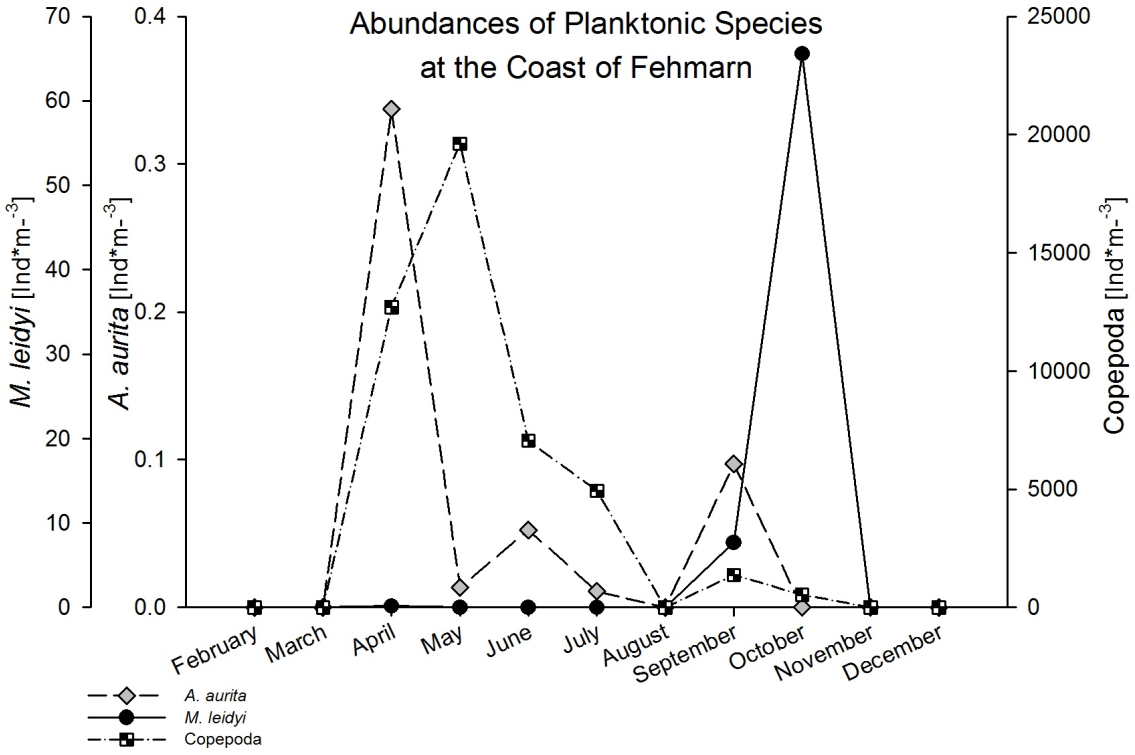


Fig. 2.4: Abundances of *M. leidyi*, *A. aurita*, and copepoda at the coast of Fehmarn in the year 2008. Five sampling locations each with 3 replicates are pooled for every month.

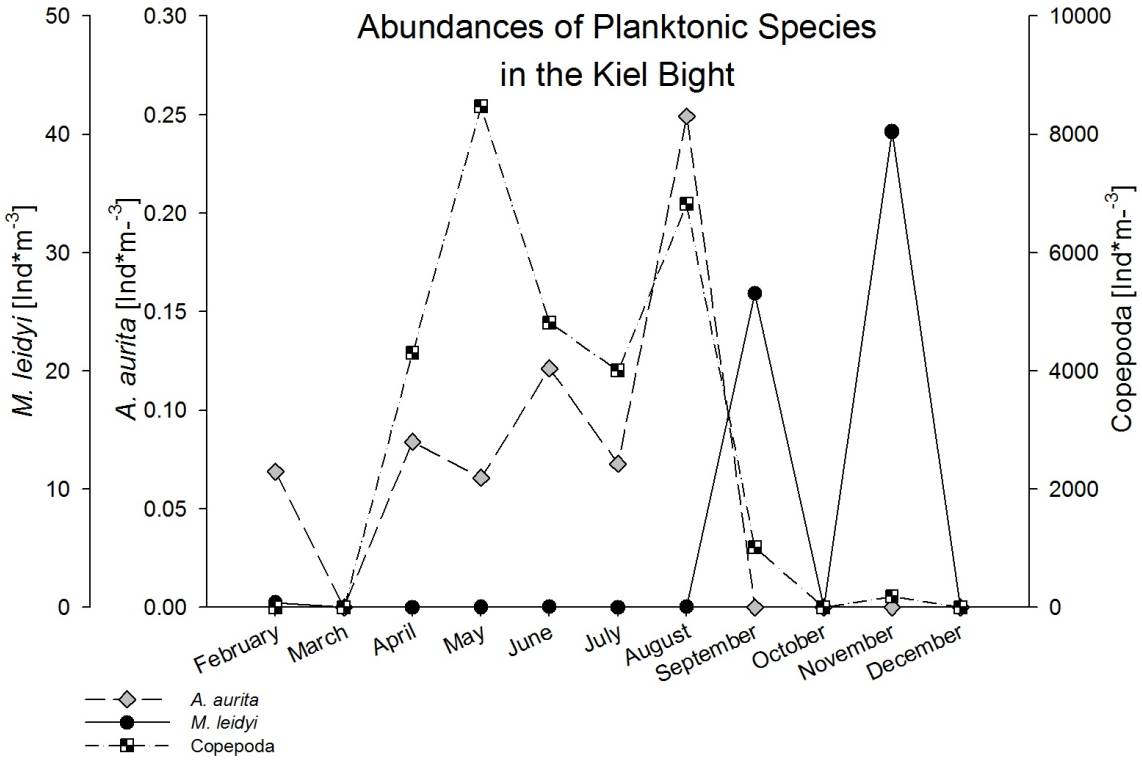


Fig. 2.5: Abundances of *M. leidyi*, *A. aurita*, and copepoda in the bight of Kiel in the year 2008. Four sampling locations each with 3 replicates are pooled for every month.

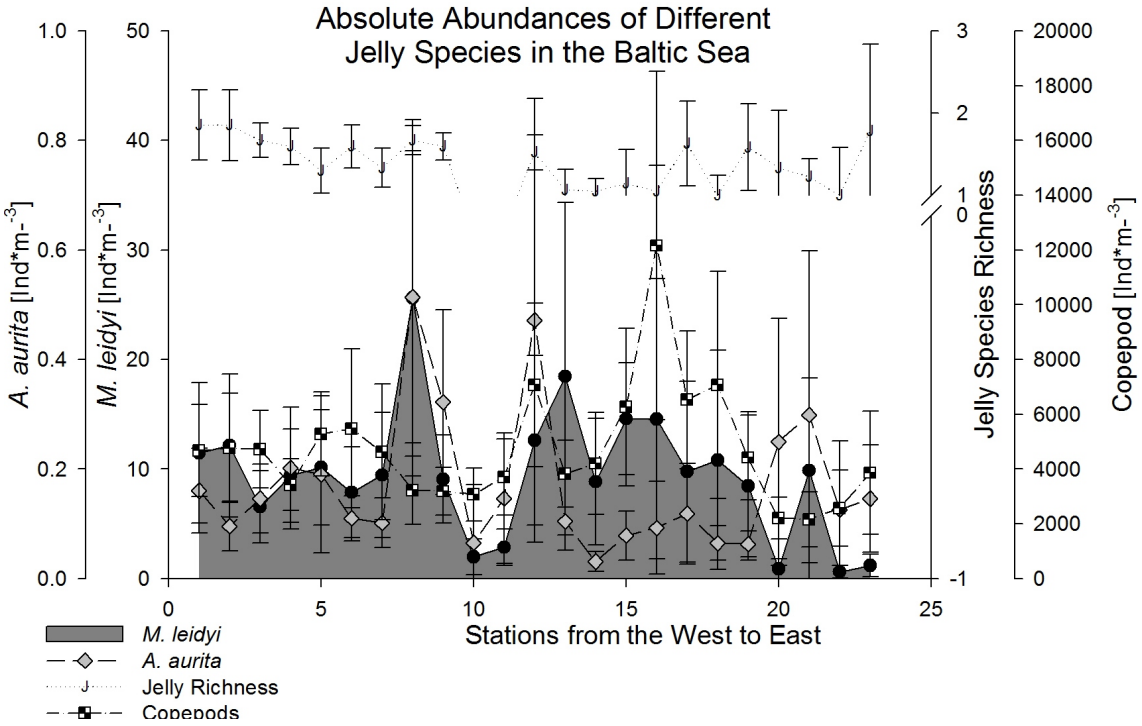


Fig. 2.6: Absolute abundances of *M. leidyi*, *A. aurita*, and copepoda in the Baltic Sea in the year 2008 plotted from west (station1') to east (station 23'). Additionally the jelly species richness, e.g. the number of jellyfish and comb jelly species, is shown. The error bars correspond to the standard errors (SE = square root (variance) / square root (n)).

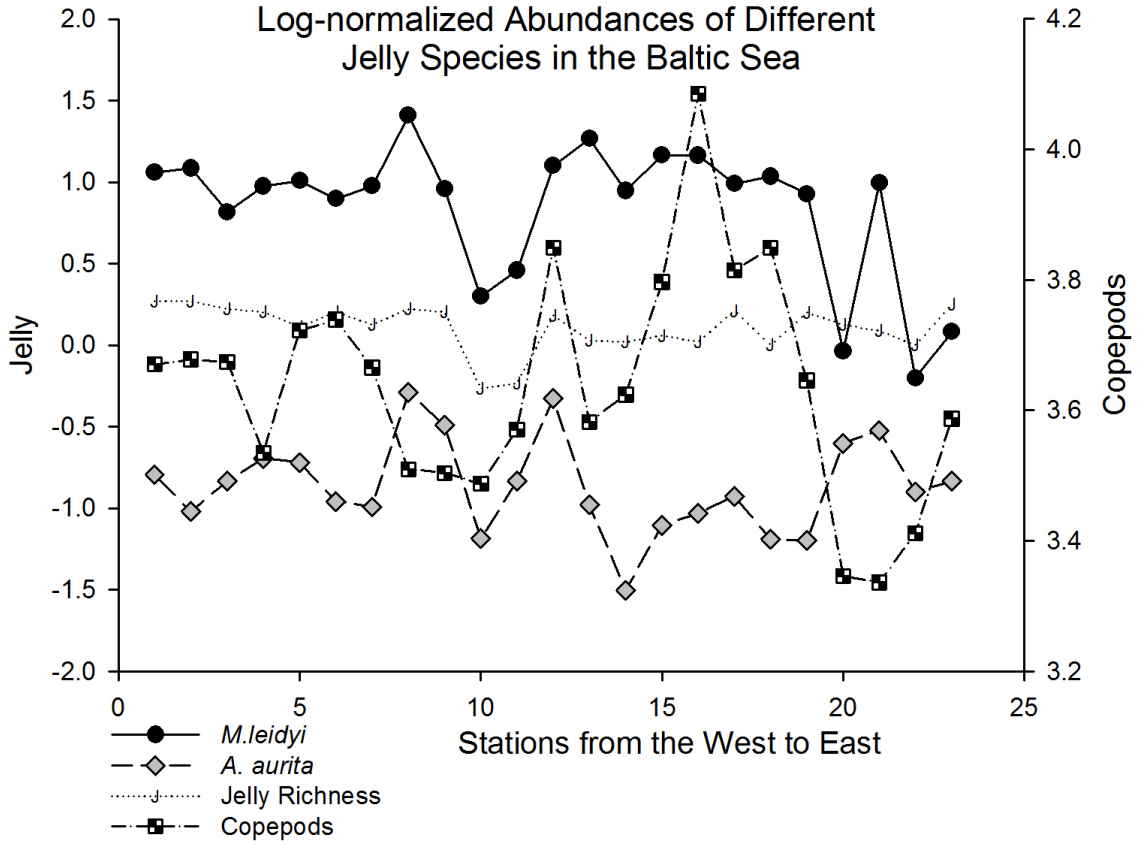


Fig. 2.7: Log-normalized abundances of *M. leidyi*, *A. aurita*, and copepoda in the Baltic Sea in the year 2008 plotted from West (station 1') to east (station 23'). Additionally the jelly species richness, e.g. the number of jellyfish and comb jelly species, is shown.

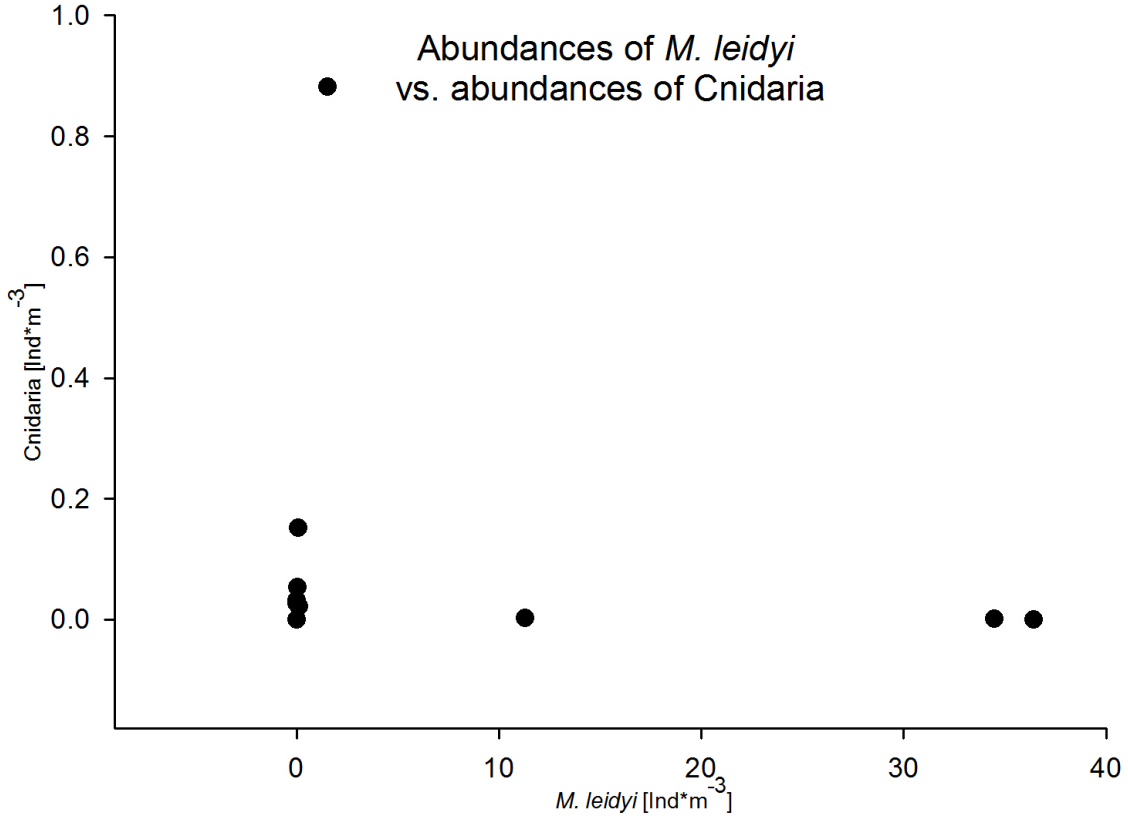


Fig. 2.8: Correlation of *M. leidyi* and all found Cnidaria, mainly *A. aurita* in the bight of Eckernförde in 2008. Every data point is pooled out of five sampling stations each with three replicates.

2.5 Discussion

The constant observations of the sea walnut during this study and the years before and afterwards led to the conclusion that a stable and self-reproducing community of *Mnemiopsis leidyi* in the central Baltic Sea already exists (Jaspers et al., 2011; Javidpour et al., 2008; Javidpour, 2006). A noteworthy observation is the same spatial preference of all jelly species, be them Cnidaria or Ctenophora, for their main blooms. Although error bars are high in figure 2.6, this trend is visible and after the log-normalization it is quite obvious (figure 2.7)

The data presented here shows similar patterns in the seasonal species succession during the year 2008 at four different sampling sites (figures 2.2 to 2.5). During springtime first copepods and *A. aurita* show the highest abundances followed by *Mnemiopsis leidyi* in autumn. Thus, no direct influences from *M. leidyi* neither to the moon jellyfish nor to the copepods should be expected. In addition the fish stocks of the sampled areas in the Baltic Sea are not likely to be affected by the sea walnut. This assumption is based on the time gap between the occurrence of fish eggs and larvae mainly in spring and early summer and the bloom of *M. leidyi* in autumn on the other hand. These findings correspond with the experiments of Jaspers et al. (2011): In laboratory experiments, eggs of the Baltic cod (*Gadus morhua callarias* L.) were exposed as nutrition to *M. leidyi* and the feeding rates were negligible. This is in clear contrast to the findings in the Black Sea, where the fish stocks and the fishery collapsed quite soon after the first occurrence of the sea walnut (Shiganova, 1998; GESAMP, 1997). Although *M. leidyi* is - at least in parts - rehabilitated nowadays (Gucu, 2002), it is still widely accepted that *Mnemiopsis leidyi* is a potential harmful invader (Zaiko et al., 2010). Thus the World Conservation Union (IUCN) places the sea walnut in its list of the world's one hundred worst invasive alien species (Lowe et al., 2004).

The interesting questions, which lie in the nearly exclusive occurrence of *M. leidyi* OR *A. aurita* cannot be answered in the content of this study (see figure 2.8). The main puzzle is the relation of cause and effect or rather the identification of oppressed species and oppressor - provided the two species are connected by rivaling relationship. Further studies would help to unravel the nature of relationship between *M. leidyi* and other jellyfish and maybe the seasonal alteration. It might well be that in spring/summer the moon jellyfish suppresses the sea walnut, whereas in autumn this pattern is inverted. Feeding experiments showed that adult *Aurelia aurita* are feeding on eggs and larvae of *Mnemiopsis leidyi* , while adult sea walnuts feed in return on eggs and larvae of the moon jellyfish (Javidpour, personal communication). Compared to data from its native area (Kremer, 1994), the peak of the bloom is switched from summer to autumn in the Baltic, which might be a hint for

feeding pressure especially in spring and summer of *A. aurita* to the sea walnut.

So far it seems that *Mnemiopsis leidyi* has established itself as a new member of the Baltic Sea species community without any negative effects for the ecosystem. The stocks of the Baltic cod are not affected so far and the other members of the planktonic community seem to have arranged themselves with the newcomer. However, it is to early to safely conclude on a final interpretation, as it has to be followed how the potential prey organisms like copepods and other small planktonic animals will cope with the rising of a new voracious predator in the medium term. And in the long term there will be even more challenges for the community: Prognoses in the light of climate change estimate a strong change in the salinity, in some scenarios up to 5 psu in the western and central Baltic Sea in the upcoming decades (MacKenzie et al., 2007; Neumann, 2010). It will be interesting how the interactions and species relationships will change under these new conditions. Taking the high tolerances against environmental conditions of *Mnemiopsis leidyi* into account, it might stretch its range of distribution in time and space at the cost of other species. Especially the potential prey organisms and the animals directly competing for food and space like the jellyfish *A. aurita* and fish (-larvae) like the Baltic cod are likely to be affected considerably.

To illustrate the fragility of the assumed newly formed species community, and the necessity to further investigate the poorly understood phenomena found in the context of this study, a small addendum shall be given. In autumn 2010/11 and 2011/12 the expected blooms of *M. leidyi* failed. This correlates with comparably low numbers of all jelly species, including *Aurelia aurita* (personal observations). Reasons for this observation remain unknown so far but one factor might have been the hard and long precedent winters and also the water currents and influxes from the North Sea, which change on an irregular basis.

3. PART 2 - SYMBIOTIC INTERACTIONS

"You Never Walk Alone"

(Richard Rodgers & Oscar Hammerstein II, 1945)

3.1 Abstract

Seawater is a dense microbial suspension with $> 10^6$ prokaryotic and $> 10^4$ eukaryotic propagules per milliliter. Hence, submerged surfaces get immediately covered by biofilm-forming colonizers upon contact with seawater. Since biofilms may reduce individual fitness through decreasing motility and attractiveness or increasing shearing stress by water currents and infection risk by pathogens, marine organisms evolved to invest energy in reducing the number of surface-colonizers, and/or tolerating settlement and biofilm-formation. Such defense mechanisms co-evolved with potentially colonizing microbes. In contrast, neozoa are confronted with novel microbial colonizers upon invading a new habitat, and are expected to be less well protected against surface-colonization. Here results of a thorough screening of the umbrella of the invasive ctenophore *Mnemiopsis leidyi* for epithelial bacteria and archaea are presented. Neither light- and electron-microscopic inspection nor PCR-screening for bacterial and archaeal DNA of 134 adult specimens from different collection sites in the Western Baltic Sea revealed any hint on the presence of prokaryotes on the comb jelly's epithelium. A limited number of bacterial associates was evident from whole-body extracts of both juvenile and adult comb jellies. Their taxonomic diversity, however, was significantly lower in adult than in juvenile specimens, suggesting a maturation of anti-microbial defense upon ontogenetic development. While the mechanisms underlying the effective defense of *Mnemiopsis* against microbial colonization remain unknown, these findings stress the suitability of Ctenophora as a basal model for interactions of metazoans with their epithelial microbiota. Based on this findings, I propose to make use of invasion events as natural space-for-time experiments on how (sym)biotic interactions change upon environmental change.

3.2 Introduction

The significance of symbiont-host interactions for ecological processes is currently receiving increasing attention. Our particular focus lies on how these interactions are shaped by environmental conditions. Symbiotic interactions, irrespective of whether the symbiont (usually the smaller partner) exerts negative (parasites and pathogens), neutral (commensalists) or positive (mutualists) effects on its host (usually the habitat of the symbiont), clearly affect the performance of at least one of the partners. To this end, environmental conditions mediate the outcome of symbiotic interactions (Steinert et al., 2000). Thus, a symbiont that exerts positive effects on its host in one environment - i.e., a mutualist - may become a parasite in another environment, or vice versa. Effects of changing environments on ecological interactions and eco-physiological processes can best be studied in "natural experiments" using organisms that are currently spreading to colonize previously unexplored habitats. Invasive species, thus, provide an excellent model for studying the effects of changing environments on symbiont-host interactions. Upon invasion of a new habitat, both partners face changes in their biotic and abiotic environment simultaneously, and symbiont-host interactions and established symbionts may change in quality and exclusiveness.

Mnemiopsis leidyi (Ctenophora) recently started invading the North and the Baltic Sea (Faasse and Bayha (2006); Hansson (2006); Javidpour (2006); Boersma et al. (2007)), after it had been reported earlier from the Black Sea and the Caspian Sea (Vinogradov et al. (1989); Studenikina et al. (1991); Shiganova (1993)). To this end, these invaders provide a model for real-time studies of ecological and physiological interactions, such as between a host and its symbionts, in the context of adaptation to, and success in a changing environment. Further, Dunn et al. (2008) and Hejnol et al. (2009) provided convincing molecular evidence for Ctenophora to represent the phylogenetic base of metazoans, being the sister group of all other metazoan taxa (see also: Wallberg et al. (2004)). Hence *M. leidyi* appears to be a valuable model for studying the very basis of the metazoan immune system and of basal mechanisms of interactions with, and control of, symbionts, be they mutualistic or parasitic, particularly so under conditions of environmental change.

Invading a new habitat, *Mnemiopsis* and any associated species that has been co-introduced have to face novel conditions with respect to both the abiotic environment and biotic interactions. Several symbionts associated with ctenophores, particularly the genus *Mnemiopsis*, have been described - such as amphipods of the suborder "Hyperiidea" (Crustacea: Amphipoda) (Sorarrain et al., (2001); Gasca and Haddock (2004)); metacercaria of representatives of three digenean trematode families (Trematoda: Digenea: Faustulidae, Lepocreadiidae, Hemiuridae) that mature

in fish predators of ctenophores were found in remarkable prevalence and infestation intensity (Martorelli, 2001); parasitic stages of the sea anemone *Edwardsiella lineata* (Reitzel et al., 2007) - but detailed information is scarce, in particular with respect to the geographical distribution of these metazoan symbionts in relation to the distribution of the host. Specifically, data on host-specificity of symbionts and on how the host is affected by the symbiotic association are essentially lacking.

Protists appear to be common symbionts of ctenophores (Mills and McLean (1991); Estes et al. (1997); Moss et al. (2001b)). According to Moss et al. (2001a), both mutualistic and parasitic, as well as purely commensalistic, protists are associated with *Mnemiopsis mccradyi*, possibly a synonym of *M. leidyi*. These protists appear to be specialized to inhabit well-defined body regions of their host - i.e., exclusively the surface of the comb plates or the ectoderm - but taxonomically related species are common as either symbiotic or free-living forms in a variety of habitats. Environmental conditions may affect the suitability of ctenophores as potential hosts (Khan (1990); Khan et al. (1993)). In particular, low salinity appears to promote infestation by protists, and - although details are essentially unknown - high infestation rates may affect Mnemiopsis performance (cf. Moss et al. (2001a)).

As it has been shown for numerous invertebrates, close associations with bacteria are common in both aquatic and terrestrial environments (for a recent review, see Dale and Moran (2006)); in some cases, high specificity on both partners' sides has explicitly been shown (e.g., Moran and Baumann (2000); Fraune and Bosch (2007); Fraune and Zimmer (2008)). Clearly, opportunistic and obligate symbionts co-exist and share the same host. So far, only one bacterial symbiont of *Mnemiopsis* (*mccradyi*) has been described (Moss et al., 2001b): rod-shaped bacteria that live inside a ciliary structure within the food groove area - but their role is entirely unknown. As for protists (see above), the effects bacterial symbionts exert on their host are mediated by environmental conditions and the resulting status of the host. Thus, interactions between *Mnemiopsis* and bacteria that are advantageous for the former within its natural range may turn to being deleterious when conditions in a newly established habitat weaken the host's performance, whereas parasitic bacteria might become useful under changing environmental conditions. In particular, encounters with potentially symbiotic bacteria that are unknown to the host from its initial habitat are of interest in this context.

Based on this, I hypothesize that symbiont-host associations of an invasive host species change in quantity and quality upon invading novel habitats. As a first approach, I aimed at comparing individuals of *M.leidyi* from its native range (U.S. Atlantic coast) and newly invaded European regions (Baltic Sea) with respect to their epithelial microbiota, since I was unable to detect any metazoan symbiont associated with *M. leidyi* in the Baltic Sea (Hammann & Zimmer, unpubl.).

3.3 Material & Methods

We sampled *M. leidyi* through bucket-capture of surface-near specimens and plankton-netting (WP2-net, 100 µm) from greater depths during ship cruises throughout 2009 at different Western Baltic sampling sites: Kiel Bight (5428'2"N, 1023'80"E; 20.07.09, 02.10.09, 30.10.09); Kiel Bight (5428'6"N, 1013'70"E; 20.07.09, 02.10.09, 30.10.09); Kiel Bight (5428'6"N, 1011'40"E; 20.07.09,02.10.09, 30.10.09); Eckernförde Bight (5450'7"N, 0938'06"E; 02.10.09); Fehmarn Island (5437'2"N, 1041'94"E; 10.08.09).

After being captured, the animals were rinsed carefully with sterile sea water and either immediately surface-swabbed for screening for epithelial symbionts, or frozen at −20°C for whole-body extracts. Swabs were stored in 98% ethanol until DNA extraction.

DNA was extracted from both surface swabs and whole-body homogenates following Henne et al. (1999) (modified by R. Schmitz-Streit; adapted for comb jelly samples by S. Hammann). In brief, 1.35 mL DNA-Extraction-Buffer (DEP) was added to 0.5-1.5 mL of the sample. After adding 5 µL 20mg/mL ProteinaseK, samples were shaken for 30 min at 37°C. Afterwards, 1.5 µL RNase (7U/ µL) were carefully mixed in. After incubation for 30 min at 37°C, 150 µL 20% SDS (sodium dodecyl sulfate) were added, and samples were inverted every 10-15 min for 2 h at 65°C. After centrifugation (12 min at 7,000 rpm; RT) the supernatant was transferred for further processing, whereas the pellet was treated again with DEP (450 µL) and 20% SDS (50 µL), inverted and incubated for 10 min at 65°C. Again, samples were centrifuged (12 min at 7,000 rpm; RT) and the supernatant was merged with the first supernatant. After adding 900 µL chloroform:isoamyl alcohol (24:1) and inversion, samples were centrifuged for 20 min at 8,500 rpm (RT). Aliquots of 750 µL of the supernatant were mixed with the same volume of isopropanol, inverted and incubated overnight at 4°C. After centrifugation (20 min, 13,000 rpm, 4°C), the pellet was resuspended in 200 µL ice-cold 70% ethanol, inverted and centrifuged for 2 min at 13,000 rpm (4°C). Subsequently, the pellet was air-dried and resuspended in 50 µL TE (Trishydroxymethylaminomethan-Ethylendiamintetraacetat) overnight.

For cleaning extracted DNA, 150 µL double-distilled water was added and mixed with 200 µL of phenol-chloroform. After shaking and centrifugation (1 min, 13,000 rpm, RT), the upper phase was treated the same way again. Then, 1/10 of the volume of 3 M sodium-acetate and three volumes of absolute ethanol were added and incubated overnight at −20°C. After centrifugation (12 min, 13,000 rpm, RT), the pellet was resuspended in 50 µL TE overnight.

Universal bacterial PCR primers (27F (5'- TG(A/G)GTTTGATC(A/C)TGG CT(C/T)AG-3') and 1492R (5'- TGG(A/C/T)TACCTTGTTACGACTT-3') Weis-

burg et al. (1991)) were used to amplify the region corresponding to positions 27-1492 of the *E. coli* 16S rRNA gene. PCR was performed with the Promega GoTaq Green Master Mix. In order to detect and amplify small amounts of bacterial DNA, PCR followed a touch-down protocol: 94°C: 3 min; ((94°C: 30 s; 65°C: 30 s (ΔT = -0.5°C/cycle)) * 24 cycles with 72°C, 90 s; 15 cycles with 94°C: 30 s, 54°C: 30 s, 72°C: 90 s); 72°C: 3 min; hold at 4°C.

The resulting PCR fragments were cloned into pGEMT vector (Promega, Madison, WI) and transformed into DH5α *E. coli* cells (Invitrogen, Karlsruhe, Germany). The Sanger-sequencing was done by the IKMB in Kiel. The sequences were edited and analyzed with the following programs: BioEdit version 7.0.9.0 (Hall, 1999), MEGA version 4.1, DNAMAN version 4.15, sequin version 9.50. Web search for related sequences of the sequenced rRNA-genes was done with the BlastSearch of the NCBI.

For the estimation of bacterial phylotype diversity, the Chao1 nonparametric richness estimator (Chao, 1984) implemented in EstimateS (version 8.2, http://purl.oclc.org/estimates) was used, treating each comb jelly specimen as a separate sample (c.f. Fraune and Bosch (2007)).

Fixation of comb jelly epithelia for electron microscopy followed Tamm and Tamm (1981). Screening of epithelial surfaces for attached prokaryotes or protists was performed at the Central Microscopy Unit of Kiel University.

Unless otherwise indicated all chemicals are purchased from Merck KGaA.

3.4 Results

Whole-body extracts (N = 14) of juvenile *Mnemiopsis* from the Baltic Sea yielded 8 different sequences of amplified bacterial 16S rRNA genes that could be affiliated to diverse bacterial classes (Fig. 3.1). Based on this, a bacterial diversity (Chao1 estimator) of 20 ± 11 (mean $\pm$ S.D.) in this category was estimated. From 30 whole-body extracts of adult *M. leidyi* (Baltic Sea) another 8 different phylotypes that belonged to either α- (3) or γ- (5) Proteobacteria (fig. 3.1) was obtained, suggesting specific host-symbiont associations with a bacterial diversity (Chao1 estimator) of as low as 8 ± 3. Remarkably, maximal one single bacterial phylotype was associated with a given adult individual, and in >85% of the screened individuals (whole-body extracts) any bacterial or archaeal symbiont could be detected (fig. 3.2). None of the found phylotypes in adults were shared with larval *M. leidyi*, and only one of them was also found in our Baltic water samples. Three larva-associated phylotypes were also detected in water samples.

As indicated by PCR-analysis of surface swabs, none of the bacteria associated with adult *Mnemiopsis leidyi* resides on the surface of the umbrella: neither bacterial nor archaeal rRNA gene sequences could be amplified from the surface of 134 adult specimens from the Western Baltic Sea. The lack of bacteria and archaea on the umbrella surface was also corroborated by electron-microscopic inspection of *M. leidyi* collected in the Baltic Sea.

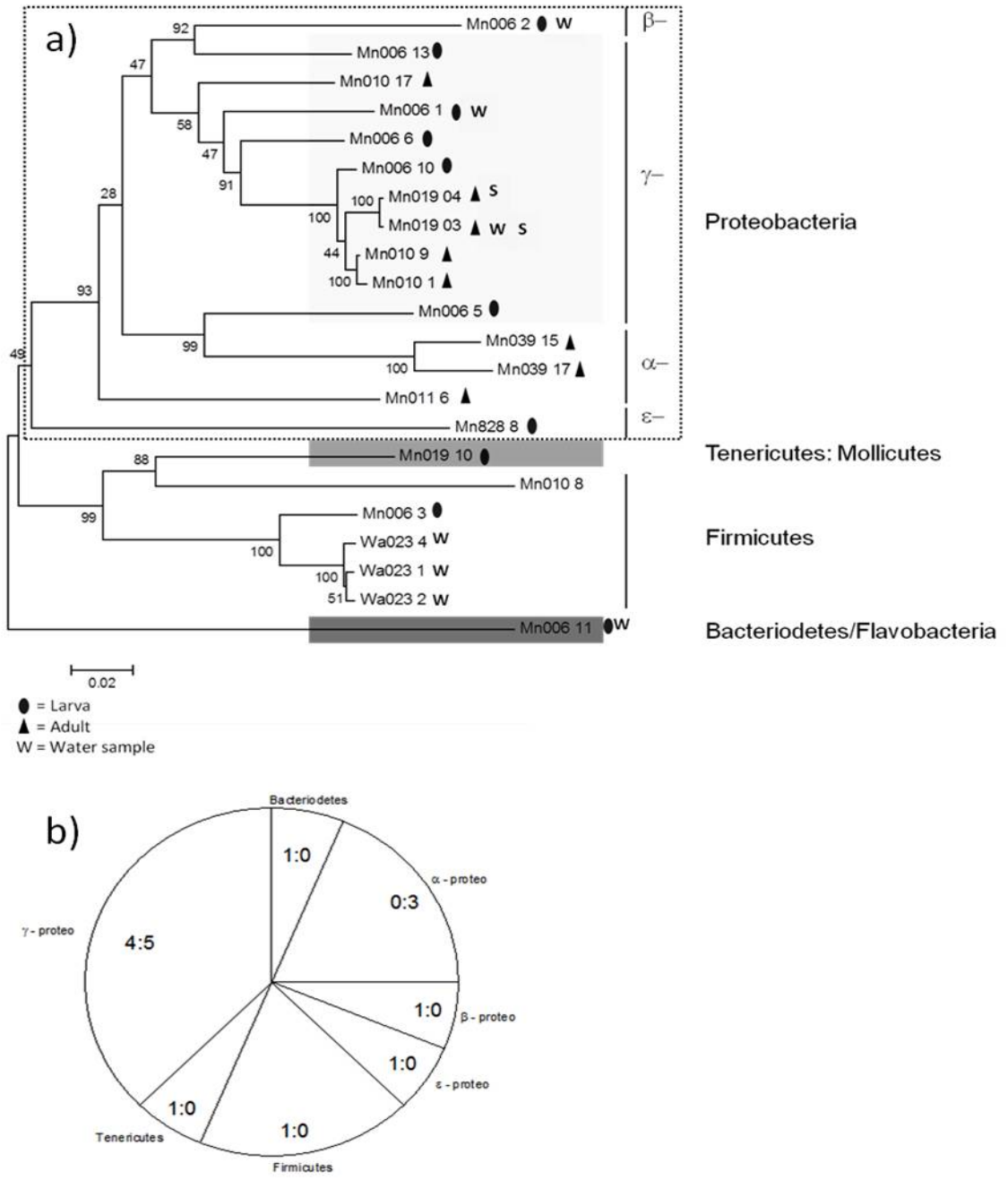


Fig. 3.1: (a) Phylogenetic affiliation of bacterial 16S rRNA gene sequences obtained from whole-body extracts of larval (ellipsoid; N = 14) and adult (triangle; N = 30) *M. leidyi*, and the surrounding water column (w). Neighbor-Joining-Tree with bootstrap 1000, generated with MEGA 4.1 Beta. (b) Summary of (a), showing the numbers of phylotypes representative of different bacterial classes that were found in whole-body extracts of larval (L) and adult (A) Mnemiopsis leidyi; data are presented as L:A

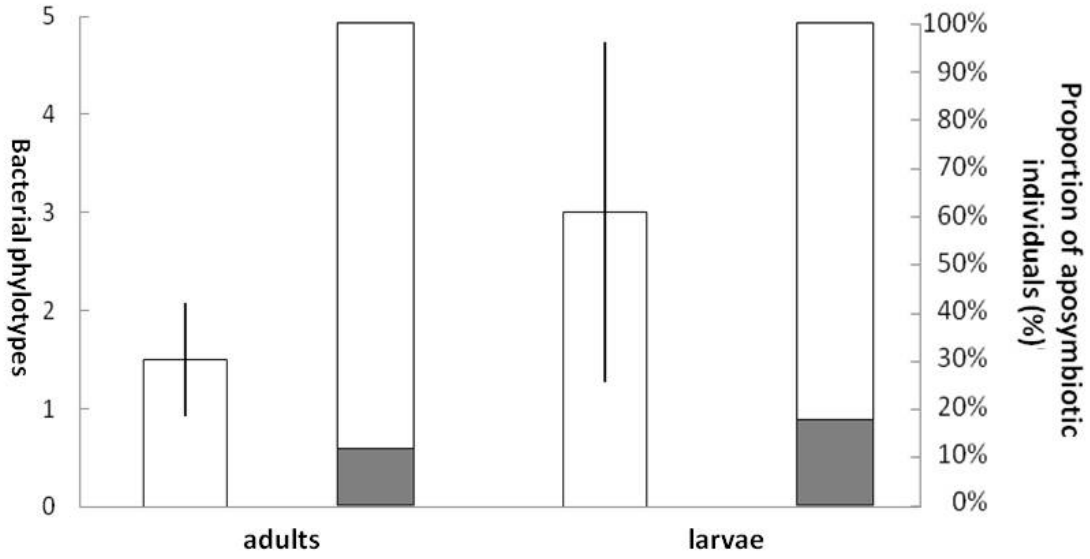


Fig. 3.2: Mean (± S.D.) number of bacterial phylotypes (left ordinate) in whole-body extracts of individual larvae (N = 14) and adults (N = 30) of *M. leidyi*, and proportion of larvae (left) and adults (right) that did (gray area) or did not (white area) harbor prokaryotic symbionts (right ordinate)

3.5 Discussion

Essentially, every submerged surface gets immediately covered by biofilm-forming bacterial, archaeal or protist colonizers upon contact with seawater. According to our careful screening of the umbrella surface of 134 adult specimens by means of PCR and electron microscopy, I conclude that the epithelial surface of *Mnemiopsis leidyi* is void of both bacteria and archaea in the recently invaded Western Baltic Sea. *Mnemiopsis leidyi* of the east coast of southern U.S. have been described to harbor specific protists. The mobiline peritrich, *Trichodina ctenophorii* (Estes et al., 1997) and small *Flabellula*-like gymnamoebae inhabit the surface of the comb plates, whereas *Vexillifera*-like gymnamoebae and *Protoodinium*-like dinoflagellates are common all over the umbrella epithelium (Moss et al., 2001a). Except for the gymnamoebae, I did not find these protists upon microscopic inspection (authors' unpubl. observation) and tentatively suggest that these protist symbionts have been lost at some stage of invading Baltic water bodies. Reasons for, and consequences of, this change in symbiotic associations remain to be studied in detail. However, it is obvious from these findings that *M. leidyi* exhibits a highly effective defense, be it chemically or mechanically, that prevents its epithelial surface from being colonized by microbial propagules from the surrounding sea water.

Considering invasion events natural space-for-time experiments on changes in biotic, and particularly symbiotic, interactions under changing environmental conditions, I hypothesize that the ability to maintain a symbiont-free epithelial surface is a key to invasion success. I view this hypothesis as an extension of the enemy-release hypothesis that had been put forward to explain why invasive species may be superior to native species (Elton, 1958) and has subsequently been tested repeatedly (c.f., Keane and Crawley (2002); Mitchell and Power (2003); DeWalt et al. (2004); Blumenthal (2006)). Here, I argue that mechanisms that keep epithelial surfaces void of symbionts help protect invaders against potential parasitic or pathogenic symbionts in their novel environment.

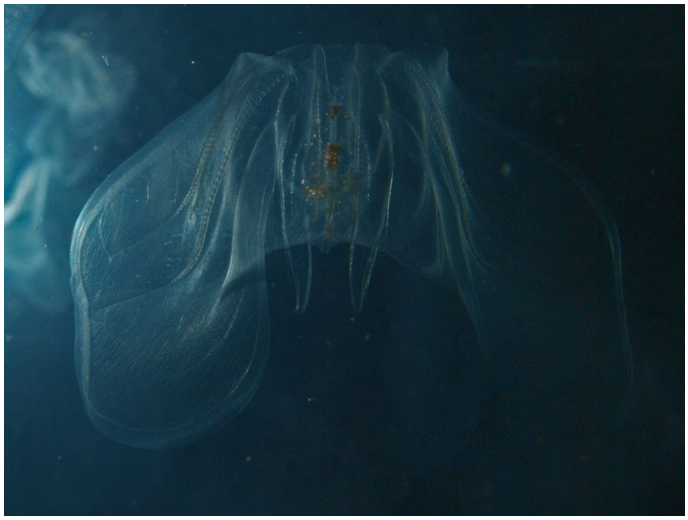
The Tens Rule for invasive species of Williamson and Fitter (1996) estimates that on average only 10% of potentially invasive species make it to the next step of successful invasion (Lockwood et al., 2007), even though conditions appear appropriate for alien species. To this end, it is tempting to hypothesize that effects of the changing environment on the community of associated symbionts controls the invasion success of non-native species. Thus, in contrast to the above extended enemy-release hypothesis, *Mnemiopsis leidyi* may be such a successful invader, because it lacks any prokaryotic epithelial symbionts that could be lost upon invading a novel habitat. These two hypotheses are not mutually exclusive, and further studies are needed to decide upon this issue.

The present data on whole-body extracts clearly indicate that there are bacteria associated with *M. leidyi*. Adults are associated with a limited diversity of bacteria (estimated 8 ± 3), whereas the diversity of bacteria associated with juveniles is about 2.5-times higher. These findings suggest that larval *Mnemiopsis* are less well defended against microbial colonization than adults, but defense mechanisms mature during ontogenetic development.

Currently, I cannot specify where the bacterial symbionts I have detected are situated. However, the obvious specificity suggests that they are either harbored inside the tissue or are specifically associated with the gut epithelium. I consider it unlikely that such low bacterial diversity reflects a bacterial community that is only loosely associated with the digestive tract and would be representative of the surrounding water column. Obviously, none of the bacterial symbionts, nor any archaea or protist is harbored on the epithelial surface of adult *M. leidyi* (in the invaded Baltic Sea). The mechanism of keeping the umbrella surface void of bacteria remains unclear - neither polar (methanol) nor apolar (hexane) whole-body extracts exhibited any antibacterial activity on settlement or growth (M. Wahl & M. Zimmer, unpubl.). Thus, it remains to be clarified which surface characteristics of the comb jelly renders the umbrella void of bacteria.

Along the same line, at present I can only speculate on evolutionary reasons for, and ecological consequences of (see above), a sterile epithelial surface. However, considering Ctenophora as an evolutionary basal representative of Metazoa, being the sister taxon of all other recent Metazoa (Dunn et al. (2008), Hejnol et al. (2009)), I propose to use *Mnemiopsis* as a model for understanding the evolutionary basis of metazoan/microbe-interactions and the metazoan immune system. On the other hand, if basal Metazoa (e.g., Ctenophora) are capable of maintaining their epithelium void of bacterial (and archaeal) colonizers, why was/is it then advantageous for their sister group (all other Metazoa) to host epithelial symbionts? Since symbiosis is commonly considered as an evolutionary motor, I hypothesize that loosing complete protection from bacterial colonization proofed advantageous through the evolutionary development of mutualistic symbiosis (that will be impossible, if colonization is prevented). Further, the evolutionary invention of developmental innovations, having led to the extant Bilateria, may in the first instance have been initiated and/or mediated by interspecific (symbiotic) interactions.

4. PART 3 - ASSESSING THE SURFACE



"Fortunately it is all on the surface."

(Oscar Wilde, Lady Windermeres Fan)

4.1 Abstract

Previous work (chapter 3) implicated the surface of *Mnemiopsis leidyi* to be void of biofilms and settling bacteria. To support these findings through microscopical techniques, a method of fixation should be established to analyze the surface of the comb jelly in its natural, undestroyed and undisturbed shape. In a second step - if the molecular findings are supported by the optical approach - it will be possible to study underlying physical and/or mechanical features of the surface epithelium that help prevent bacterial settlement. Chemical features can not be detected this way.

Unfortunately the laboratory culture of *M. leidyi* went extinct during moving laboratory from Helmholtz Centre for Ocean Research Kiel (GEOMAR) to the Biological Centre, CAU Kiel in 2011, and in addition the bloom in autumn 2011 failed, both for unknown reasons. Based on that the experiments could not be finished so far. Most of the results are not satisfying, yet. But there is a promising method, which already led to good pictures of the surface. Studies will be continued as soon as fresh living animals are available.

4.2 Introduction

Previous work - chapter 3 in this thesis - discovered the astonishing fact that the surface of the comb jelly *Mnemiopsis leidyi* is void of living bacteria. Beside biological and ecological questions such a finding provokes - Is a sterile surface a key to invasive success? What are the benefits and costs for investing energy to avoid surface settlement? What are the physiological, developmental and evolutionary consequences of keeping a surface clean? - a more basal question is arising: How does the comb jelly *M. leidyi* keep its surface not just clean but sterile? To face this last question and to support the molecular findings, I aimed at developing an optical approach.

As mentioned above, the first point in this approach was to make sure that there are really no settling bacteria on the surface of the sea walnut. In the molecular tests so-called universal bacterial primers Weisburg et al. (1991) were used. However, these universal primers do not detect all bacteria (for further discussion see Daims et al. (1999)). This point is of special relevance here, because the probes were - of course - designed on the basis of so far known bacterial 16sRNA sequences; and there are more than 1000-times more unknown than known bacteria.

The second point, if the surface turns out to be really sterile, is to answer the question for the underlying mechanisms. In principal two different mechanisms are conceivable to keep a surface clean: on the one hand a (bio-)chemical solution via antibiotics, antimicrobial peptides and/or other substances or molecules; on the other hand a physical/mechanical solution might be possible. The optical approach focuses on this last hypothesis.

Because of their extremely fragile body structure comb jellies are difficult to handle and up to now there was no known method leading to satisfying fixation results. Satisfying means here to get good reproducible pictures of the undisturbed surface and its structures. Some of the known and established protocols for jellyfish and other planktonic species and some own ideas were tested and tried to adapt to comb jellies.

All the herein presented tests were only possible because of the generous help and support - with materials, laboratory space, knowledge, and motivation - by Prof. Dr. Stanislav N. Gorb and his working group, especially Marie-Christin Klein.

4.3 Methods of Fixation and Preparation

Unless otherwise indicated all chemicals are purchased from Merck KGaA.

4.3.1 Method 1 - Autofluorescence

To stain the surface and possibly attached bacteria via FISH or similar methods, it is necessary to know the degree of autofluorescence of the comb jelly *Mnemiopsis leidyi*. The observations were done with a fluorescence microscope (Zeiss Axiplan, Germany) in combination with an Axiocam MRc and a mercury arc lamp (HBO 100W, Carl Zeiss, Germany). As figures 4.1, 4.2, and 4.3 indicate, *M. leidyi* exhibits very low autofluorescence. It was necessary to add an additional standard light source to get a picture because of the absence of any detectable autofluorescence.

In comparison, Cnidaria are showing a strong autofluorescence, rendering further staining quite difficult or even impossible (Rebecca Metzger, personal communication). The fact of the low autofluorescence allows - in theory - the further processing with FISH or other fluorescence based staining methods.

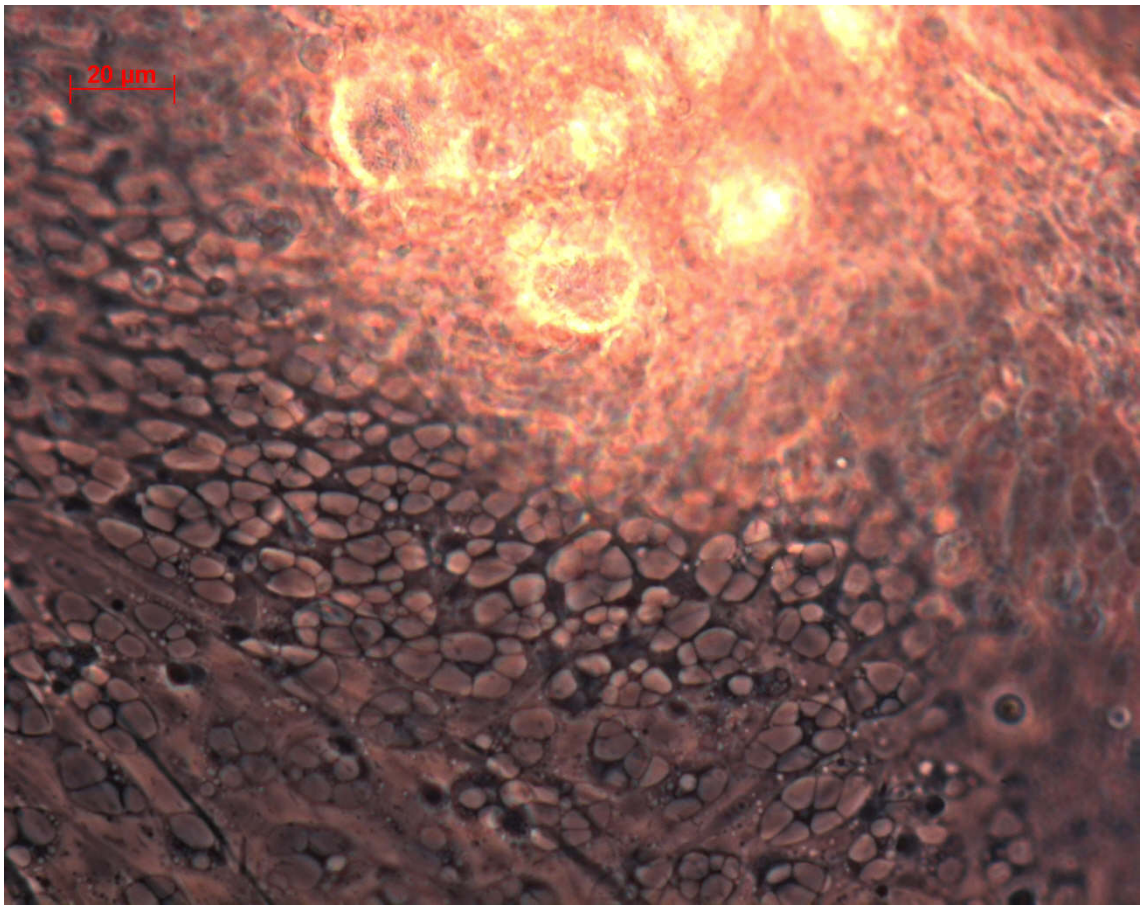


Fig. 4.1: Photo of *M. leidyi* with 50x optical enlargement. The sample is cut from a fresh living animal and analyzed without further preparation. Light source for autofluorescence is a mercury arc lamp (HBO 100Wn, Carl Zeiss, Germany); used filter set: 365/12nm, FT395nm, LP397nm, 450-490nm, FT510nm, LP520nm (blue filter), 545/25nm, FT570nm, LP590nm (green filter). No autofluorescence is detectable as shown on this picture exemplarily; to get a picture a normal light source is added.

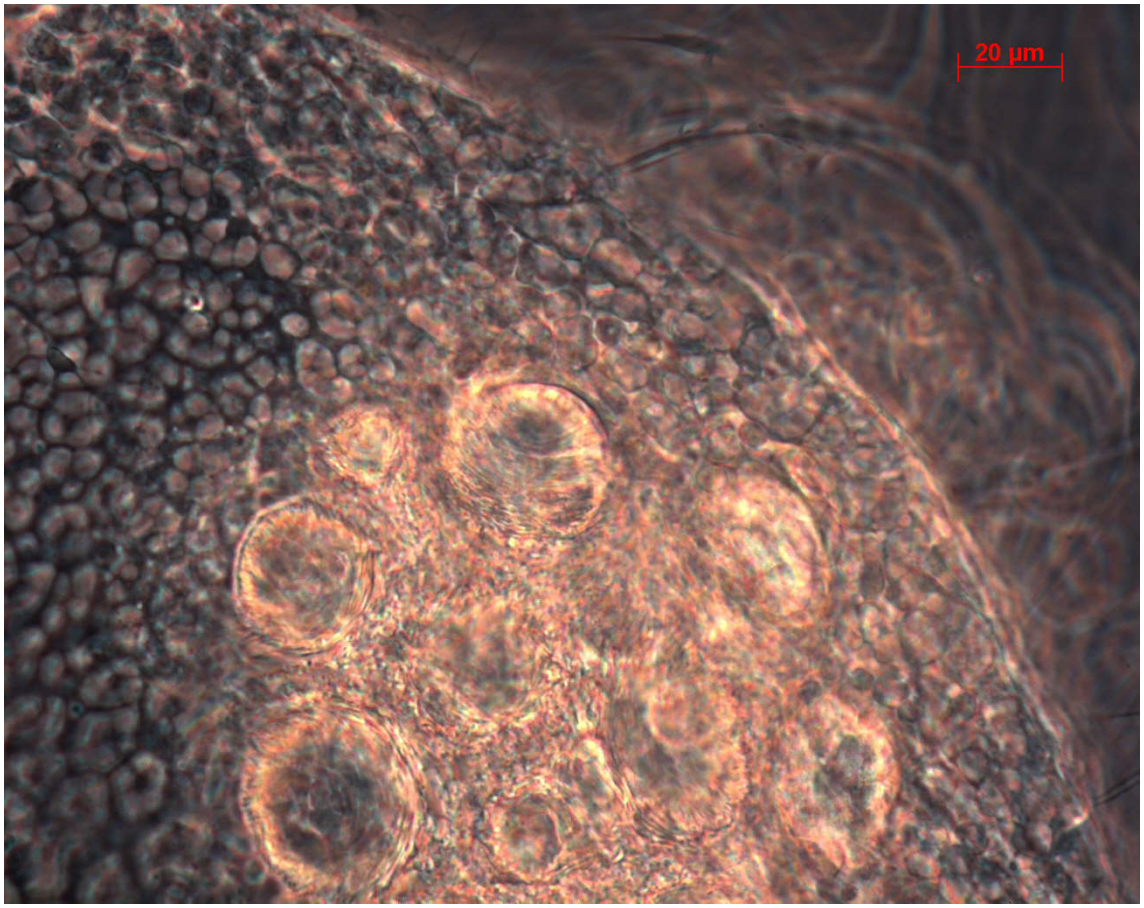


Fig. 4.2: Photo of *M. leidyi* with 50x optical enlargement. The sample is cut from a fresh living animal and analyzed without further preparation. Light source for autofluorescence is a mercury arc lamp (HBO 100Wn, Carl Zeiss, Germany); used filter set: 365/12nm, FT395nm, LP397nm, 450-490nm, FT510nm, LP520nm (blue filter), 545/25nm, FT570nm, LP590nm (green filter). No autofluorescence is detectable as shown on this picture exemplarily; to get a picture a normal light source is added.

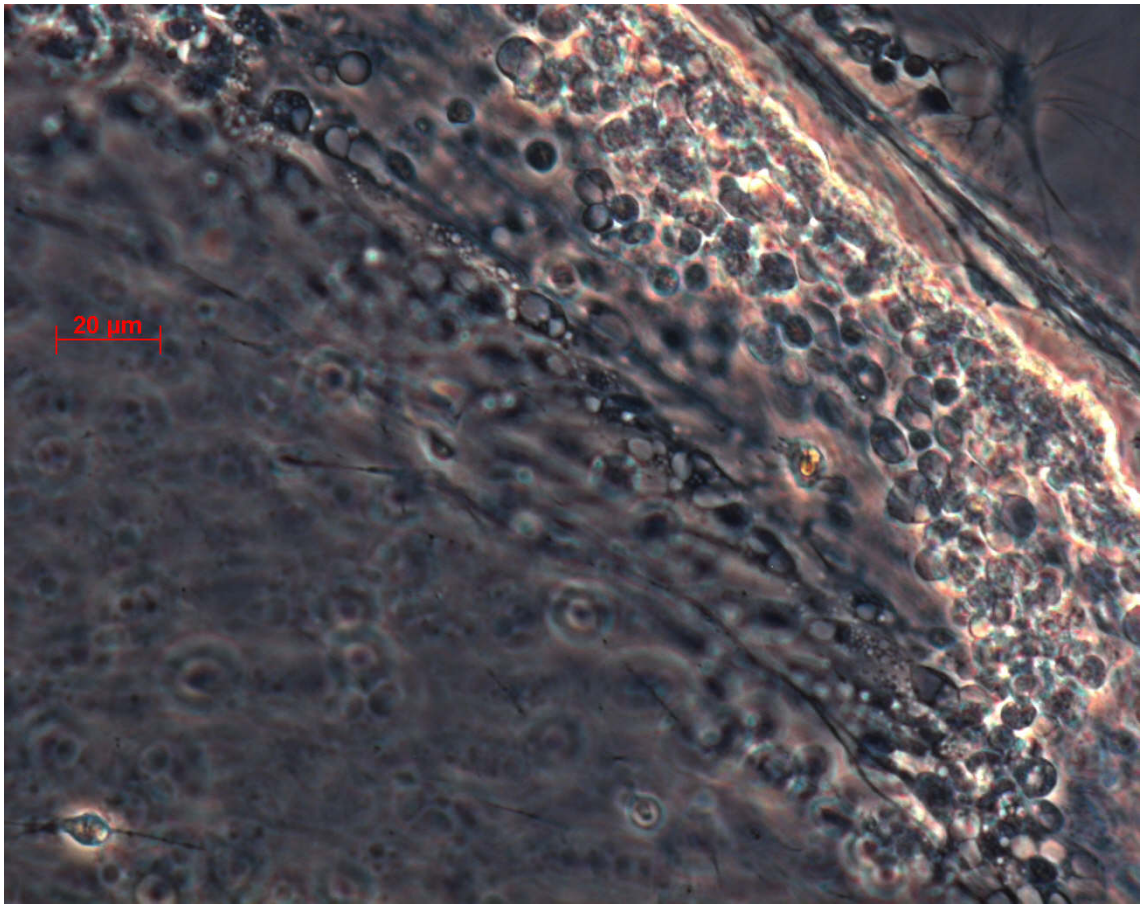


Fig. 4.3: Photo of *M. leidyi* with 50x optical enlargement. The sample is cut from a fresh living animal and analyzed without further preparation. Light source for autofluorescence is a mercury arc lamp (HBO 100Wn, Carl Zeiss, Germany); used filter set: 365/12nm, FT395nm, LP397nm, 450-490nm, FT510nm, LP520nm (blue filter), 545/25nm, FT570nm, LP590nm (green filter). No autofluorescence is detectable as shown on this picture exemplarily; to get a picture a normal light source is added.

4.3.2 Method 2 - Drying

In this series of experiments individuals of *Mnemiopsis leidyi* were dried in different variations each with 3 to 4 replicates. Before the drying started every comb jelly was carefully rinsed with ultrapure water to wash away attached seawater to minimize negative effects of crystallizing salts during the process of drying.

The first approach was to place the jellies on two different kinds of glass slides. One sort with a plane surface and one with a hollow grinding. Figure 4.5 shows that although the specimen were rinsed with ultrapure water the surface was covered with salt crystals and the tissue was disrupted either by the crystals or by shearing forces resulting from varying speeds of water loss in the different cells of the tissue or by a combination of these two processes.

To avoid the shearing forces and minimize crystallization in a next step the animals were placed on three different kinds of filter materials, again each with three replicates. The filters have been standard coffee filter paper, fiber glass and Anodiscs with a pore diameter of 0.2 µm. The filters were placed on molecular sieve to guarantee a constant and complete drying process. The experimental design is shown in figure 4.4.

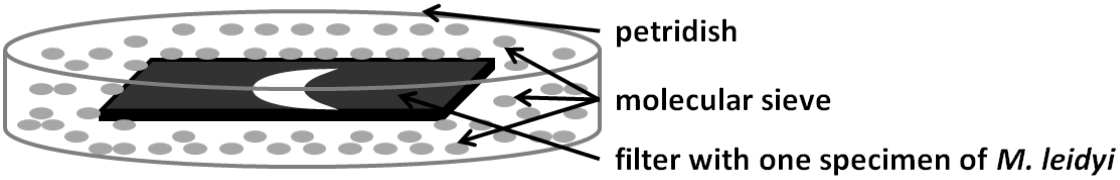


Fig. 4.4: Schematic setting of *M. leidyi* on filter drying experiments

The standard filter and the glass fiber filter (figures 4.6 and 4.7) did not lead to satisfying results. Even on high magnifications it is difficult to distinguish areas covered with *M. leidyi* or not. The very thin epithelium layers followed every anomaly of the filter surface or dissolved completely (figure 4.7).

The "AndoDisc-approach" does not lead to better results, see figures 4.8, 4.9. In figure 4.8 the filter is completely covered with salt crystals. To get rid of these the sample was carefully rinsed with ultrapure water. This procedure removed most of the remaining animal, too. Although something remains which might be - fully speculative - a nerve cell or a muscular fiber (figure 4.9).

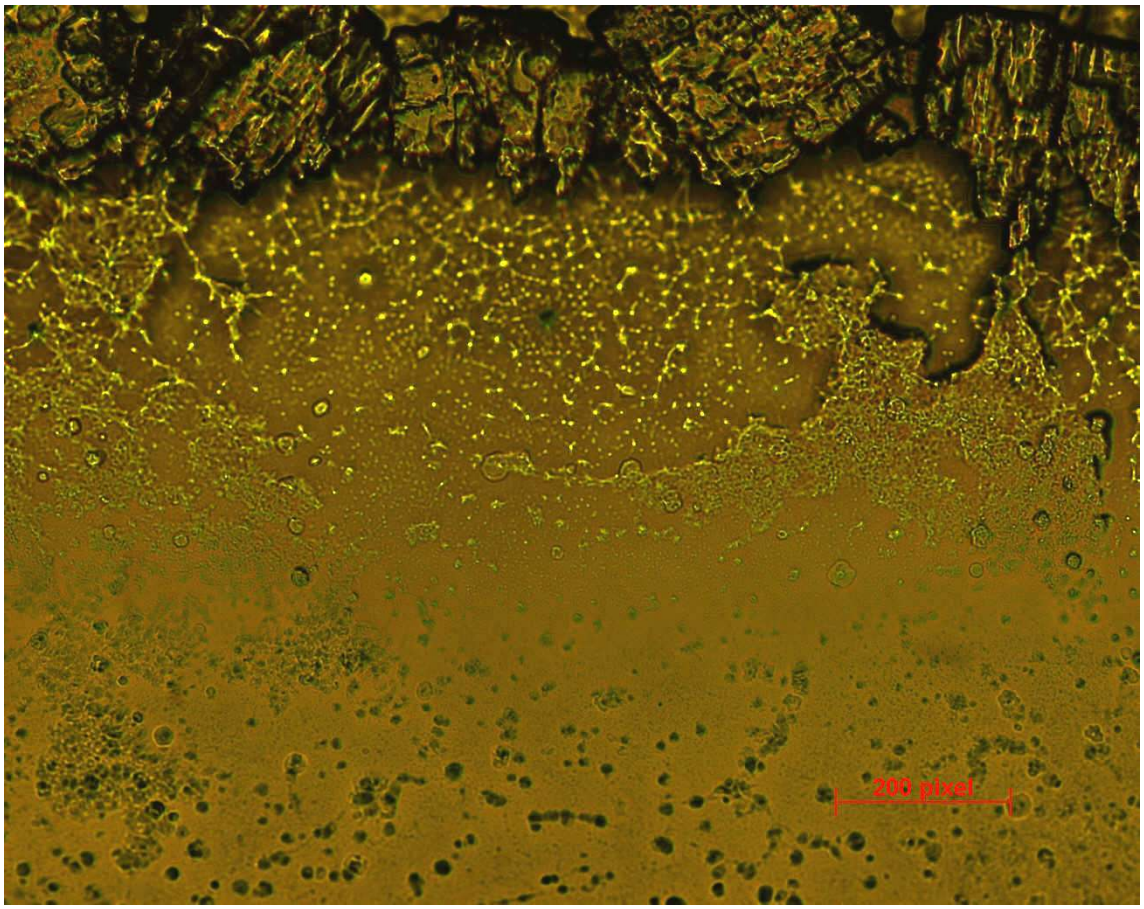


Fig. 4.5: Light microscopic picture of *M. leidyi* after drying on a glass slide; optical enlargement: 100x. No clear structures are detectable, except the salt crystals in the upper part. In the lower part rests of the mucus and parts of the mesogloea can be seen.

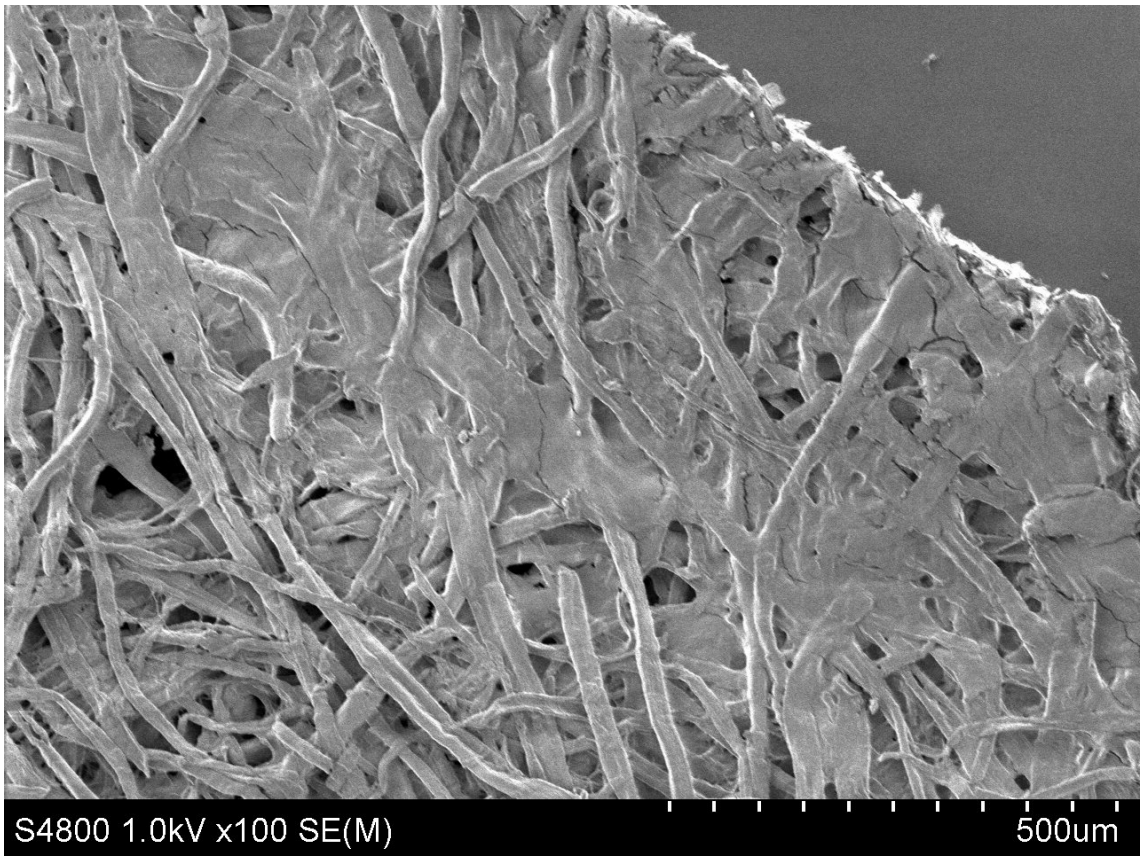


Fig. 4.6: SEM picture of *M. leidyi* dried on a glass fiber filter; optical enlargement: 100x. A thin layer -maybe of mucus- cover the fibers of the filter. Parts or cells of the animal are not distinguishable.

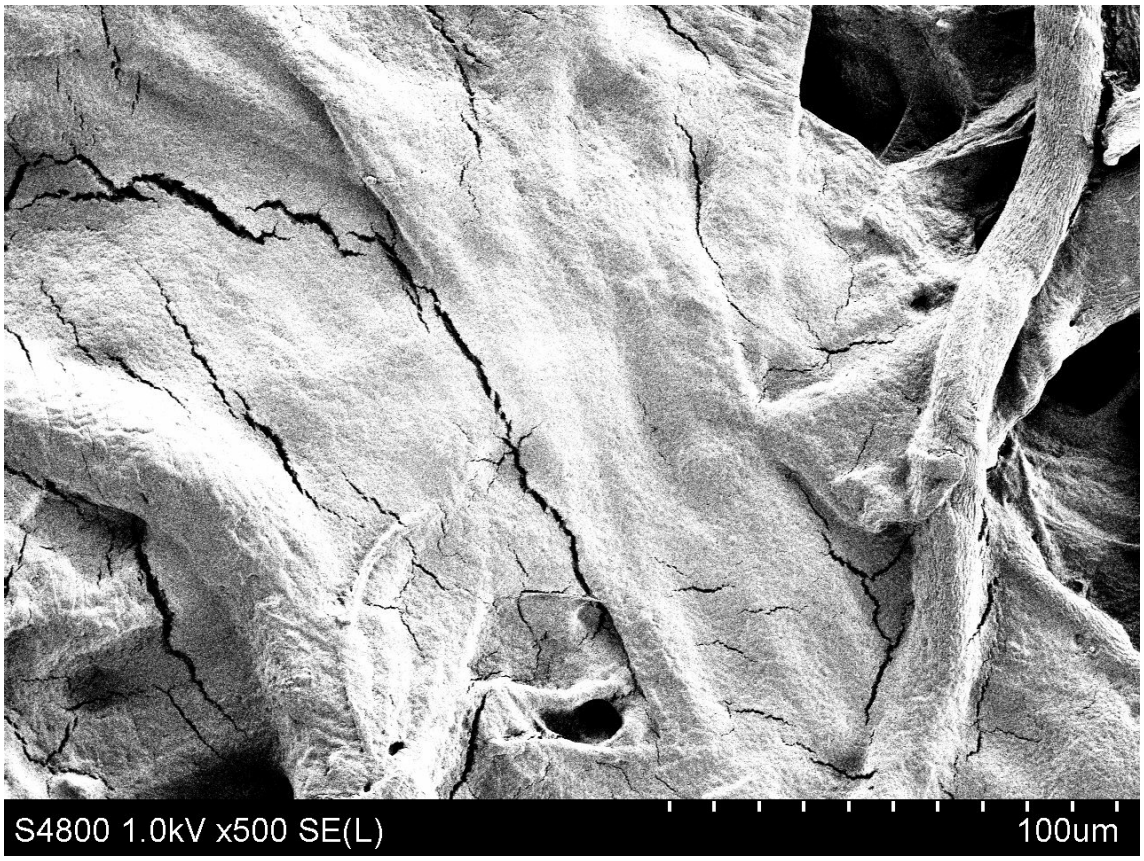


Fig. 4.7: SEM picture of *M. leidyi* dried on a glass fiber filter; optical enlargement: 500x. A thin but dense layer of mucus covers the fibers of the filter. Parts or cells of the animal are not distinguishable.

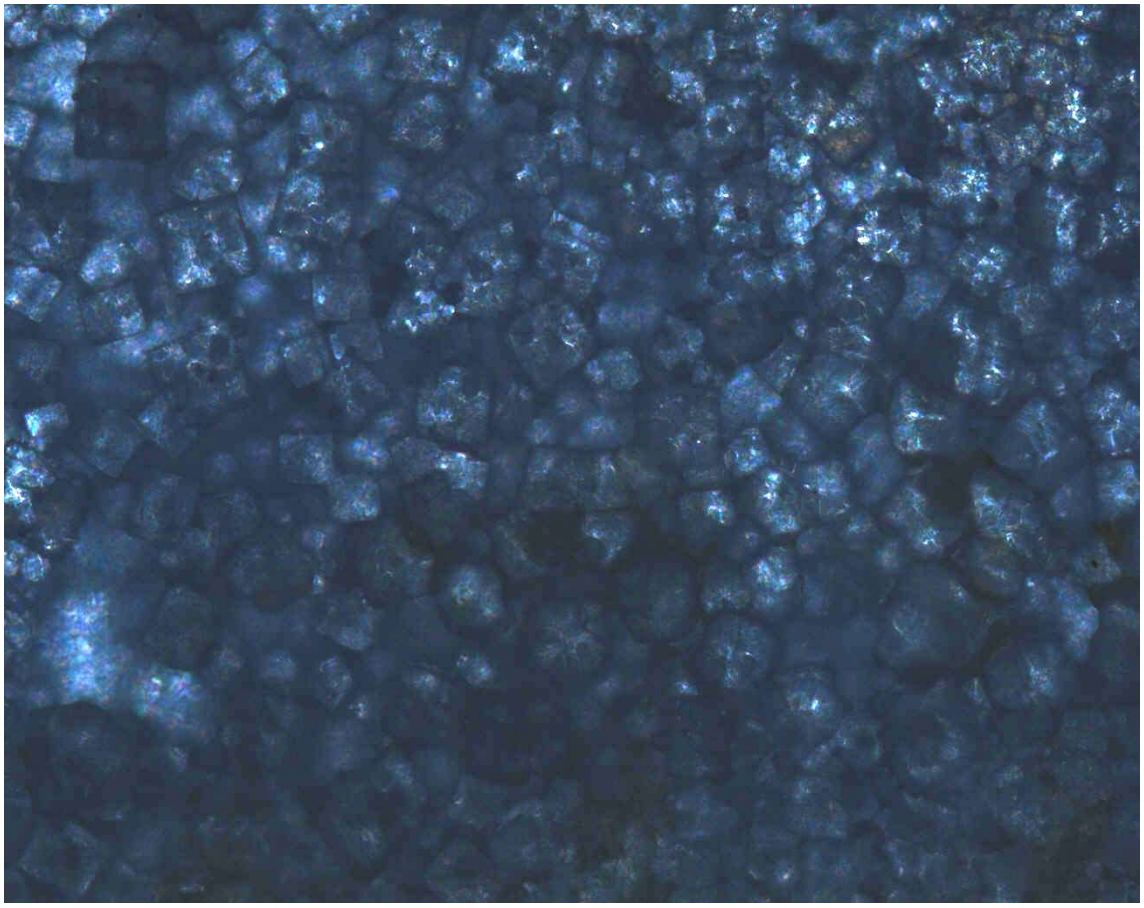


Fig. 4.8: Light microscopic picture of *M. leidyi* dried on a AnoDisc filter; optical enlargement: 400x. The whole area is covered with salt crystals; identification of other structures is not possible.

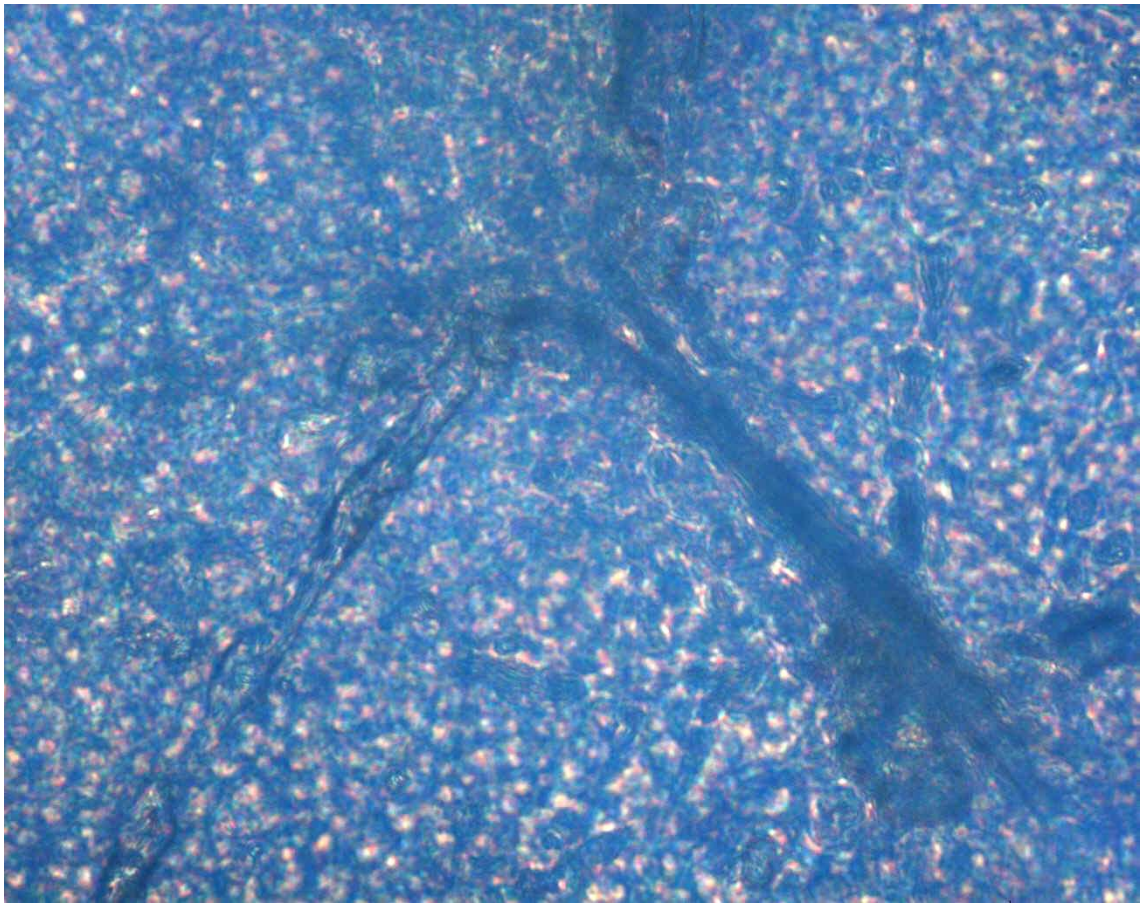


Fig. 4.9: Light microscopic picture of *M. leidyi* dried on a AnoDisc filter; optical enlargement: 400x. By rinsing with ultrapure water additionally to get rid of the salt crystals all cell structures were destroyed.

4.3.3 Method 3 - Staining with methylene blue

The goal of this method was to stain the bacteria attached to the surface of *Mnemiopsis leidyi* with methylene blue using fresh living adult animals. Methylene blue is an alkaline dye which is staining negatively charged structures like the nucleus and chromatin structures. After staining for one minute the animal was dried at a glass slide, which was placed in a petri dish filled with molecular sieve to guarantee a complete drying and to avoid re-wetting by air-humidity (see figure 4.4). The results are visible in figures 4.10 and 4.11. The animal disappeared almost completely during the staining process itself and it seems only the mucus, some combs, and/or some parts of the mesogloea remained long enough to be placed on the glass slide. In the lower left corner of figure 4.10 a structure is shown which might be the rest of one of the comb rows.

For further tests it might be advisable to adjust the dye to the osmotic capacity of sea water or the animals, respectively.

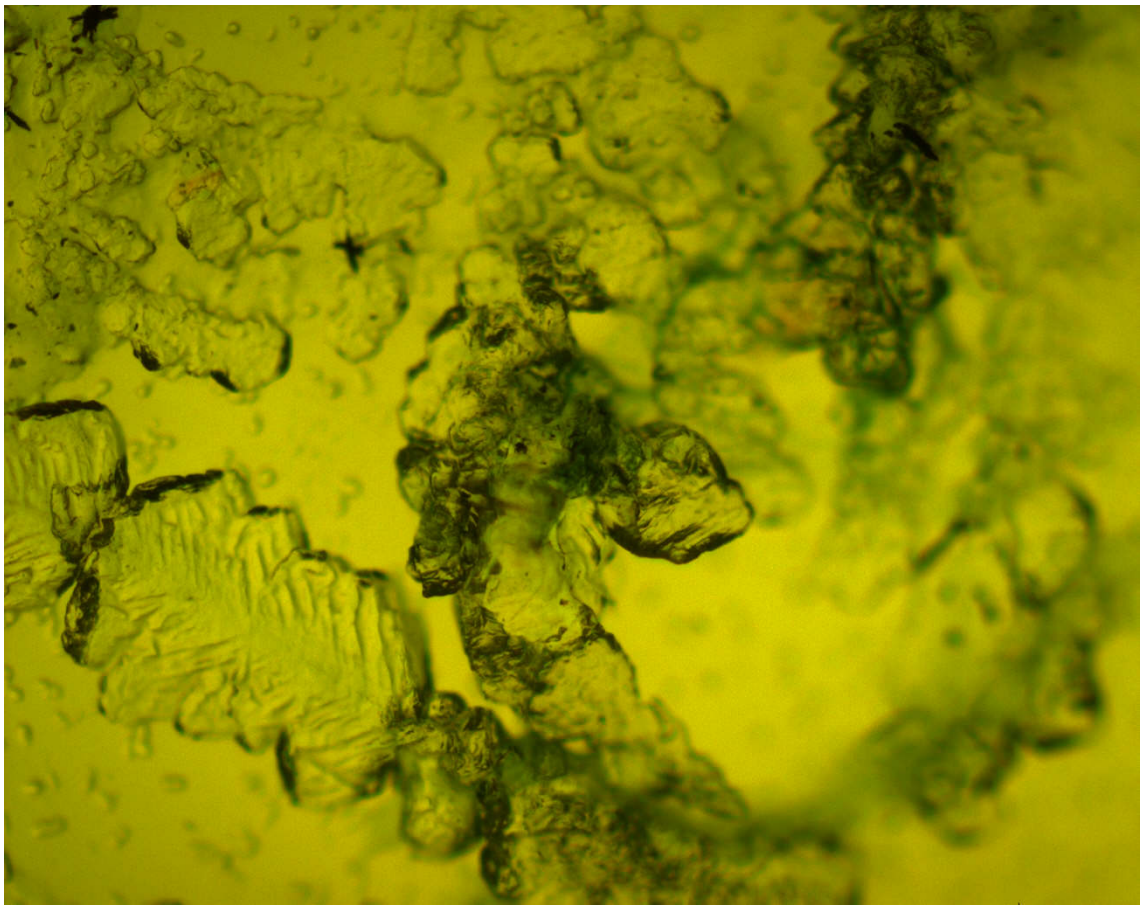


Fig. 4.10: Light microscopic picture of *M. leidyi* after staining with methylene blue and drying on a glass slide; optical enlargement: 50x. In the lower left corner the rest of a comb row can be identified. It is strongly covered with crystallized salts. This salt crystals are found all over the picture and are the only remaining structures of the animal.

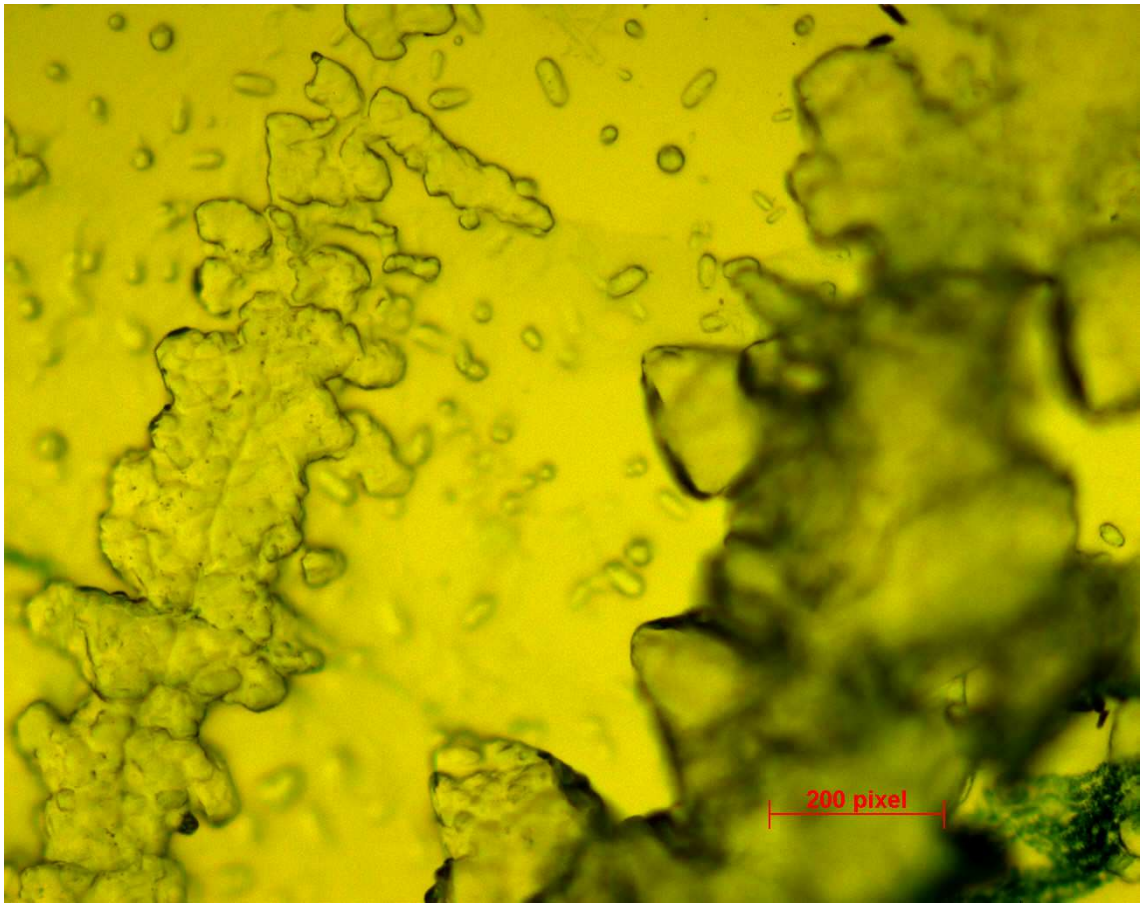


Fig. 4.11: Light microscopic picture of *M. leidyi* after staining with methylene blue and drying on a glass slide; optical enlargement: 100x. Only salt particles and single desultory cells can be seen.

4.3.4 Method 4 - Gram's staining

The commonly known method of Gram's staining - also known as Gram's method - was tested for its aptitude to visualize the bacteria potentially attached to *Mnemiopsis leidyi*, on the inner or outer tissue layers. The method follows the standard procedure (see e.g. Mulisch and Welsch (2010)). The only exception concerned the differentiation fluid: normally acetone or ethanol are used. In order to avoid the destruction of the material a 1:1 mixture of acetone and ethanol was used. The following materials were used:

- crystalviolet solution (dissolve 0.8 g ammoniumoxalate in 80 ml distilled water; dissolve 2.0 g crystalviolet in 20 ml 96% Ethanol; mix both solutions)
- Lugol's solution (dissolve 2.0 g potassiumiodide in 5 ml distilled water; add 1.0 g iodine; fill up with distilled water to 300 ml)
- 1:1 acetone: ethanol ($\geq$ 96%)
- carbolfuchsin solution (dissolve 1.0 g basic aniline red in 10 ml 96% Ethanol; liquefy 5.0 g phenol crystals by warming carefully and dissolve in 100 ml distilled water; mix both solution and and dilute the result 1:10 with distilled water)
- ultrapure water

The preparation followed the standard protocol; shortly summarized:

- pipet some crystalviolet solution onto the sample
- wait 2-3 min
- remove solution
- rinse with ultrapure water
- pipet Lugol's solution onto the sample
- wait 1 min
- rinse thoroughly with ultrapure water
- pivot the glass slide with the attached sample in the differentiation fluid until no color clouds can be seen anymore
- rinse thoroughly with ultrapure water

- counterstain with carbolfuchsin
- wait 1 min
- rinse thoroughly in ultrapure water
- dry and cover the sample

As it turned out the method is not working in this case. The comb jelly totally disappears during the contact with the differentiation liquid. It was tested in various mixing ratios (from 1:10 to 10:1) of ethanol and acetone and with the pure substances as well. The result was always the same.

Maybe it is somehow possible to fixate *M. leidyi* before the usage of the Gram-staining method possibly via the TechnoVit 9100 kit (a synthetic embedding system based on methyl methacrylate; Heraeus Kulzer Technik), but more animals are needed for further tests.

4.3.5 Method 5 - Fixation protocol for electron microscopy

For fixation for the electron microscopy the following recipe was used. It is based on a method given in Tamm and Tamm (1981) and modified by Anthony Moss (Associate Professor and Coordinator, Marine Biology; Biological Sciences, Auburn University, USA) and myself as follows:

Required materials:

- 5% paraformaldehyde
- 500 mM solution of cacodylate buffer
- Ethanol (10%, 30%, 50%, 70%, 90%, 100%)
- sterile seawater
- osmiumtetroxyd 4%
- osmiumtetroxyd 1%
- glutaraldehyde solution 25%
- 50 mM cacodylate-seawater

Store all materials on ice and prepare 25 ml of fixation solution in a sterile 50 ml Falcon tube:

- 5 ml of 5% paraformaldehyde (final concentration: 1%)
- 1 ml of 25% glutaraldehyde (final concentration: 1%)
- 4 ml of sterile seawater
- 6 ml osmiumtetroxyd (final concentration: 1%)
- fill up 25 ml with cacodylate-seawater
- swirl

After the preparation of the fixation solution, bigger animals should be cut into pieces carefully. Smaller individuals can be used without cutting. For our approach smaller individuals were preferred to avoid artificial surface destruction by cutting.

Fixation process:

- add fixation solution to tissue pieces
- fix for 1 hour or until tissue color is changing
- wash 3 to 5 times in cacodylate-seawater
- post fix in 1% osmiumtetroxyd for 1 hour
- wash 3 times in seawater
- wash 3 times in ultrapure water

After the fixation process the material should be stored in a fridge until dehydration or should be dehydrated directly:

- 10% Ethanol for 30min
- 30% Ethanol for 40min
- 50% Ethanol for 40min
- 70% Ethanol for 40min
- 90% Ethanol for 40min two times
- 100% Ethanol for 40min three times (last time also possible over night)

After the dehydration the samples can be stored in 100% Ethanol at 4 °C until critical point drying.

In the following step the material is sputtered and is ready for scanning electron microscopy (SEM). We used a Zeiss DSM 940.

The results of this approach are not satisfying (see figures 4.12, 4.13, and 4.14). In principal the method leads to pictures of high quality but the drying process leads to ruptures, up and down folding, and other artificial disturbances. It is a useful method for studying cilia or inner structures at the edges of the fractures but the goal to get a picture of an undisturbed and intact surface tissue could not be achieved.

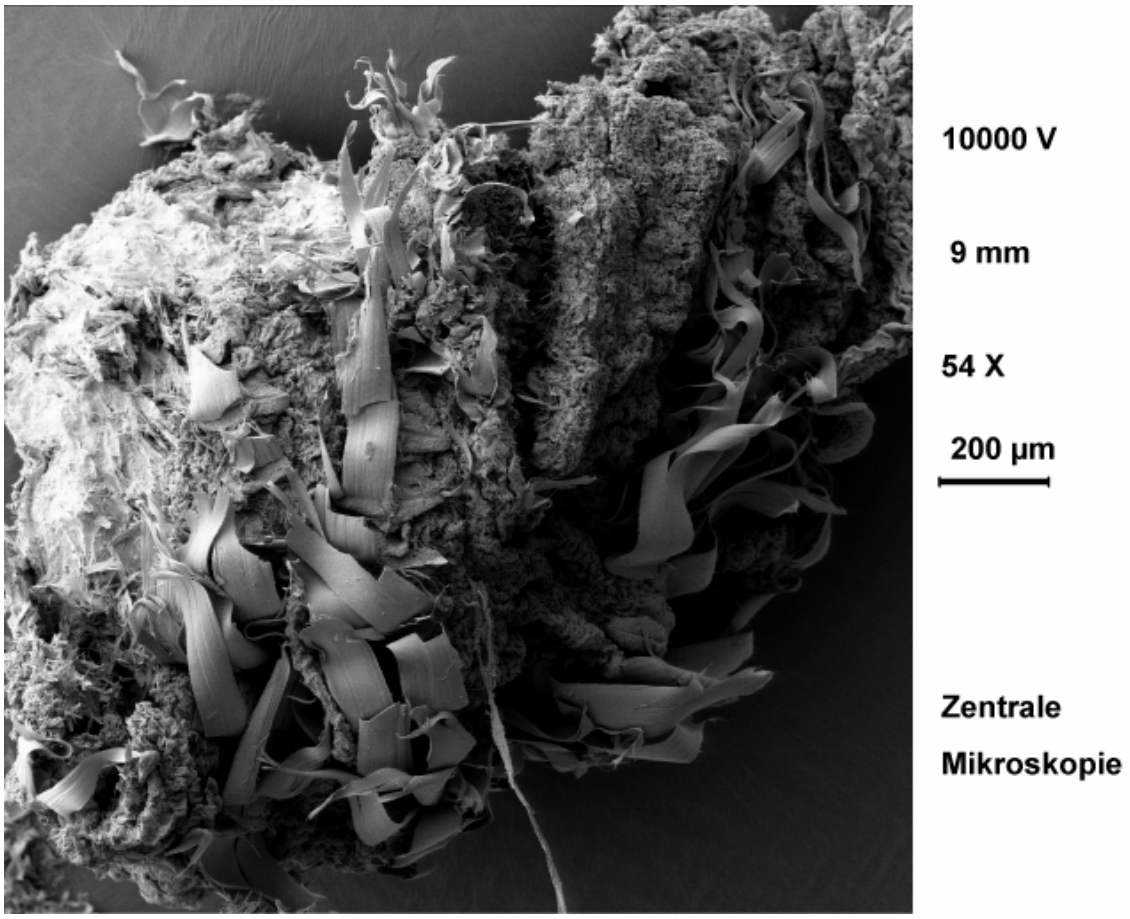


Fig. 4.12: M. leidyi after SEM-fixation. A site of surface fracture is shown, 54x optical enlargement. The arrangement of the cilia in rows is apparent, other structures are not clearly distinguishable.

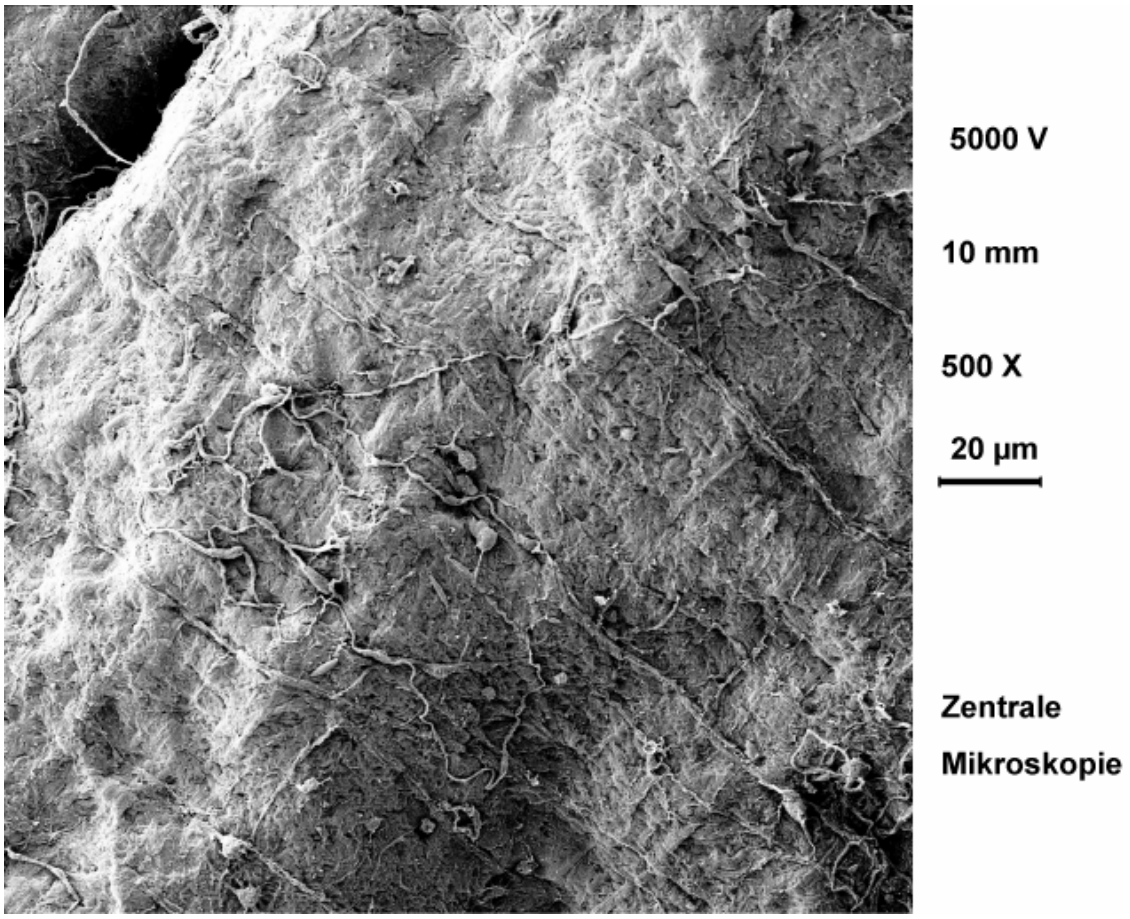


Fig. 4.13: M. leidyi after SEM-fixation. View on surface with 500x optical enlargement. The outer epithelium is disintegrated, so inner fragmentations, structures of the mesogloea, and embedded cells can be seen.

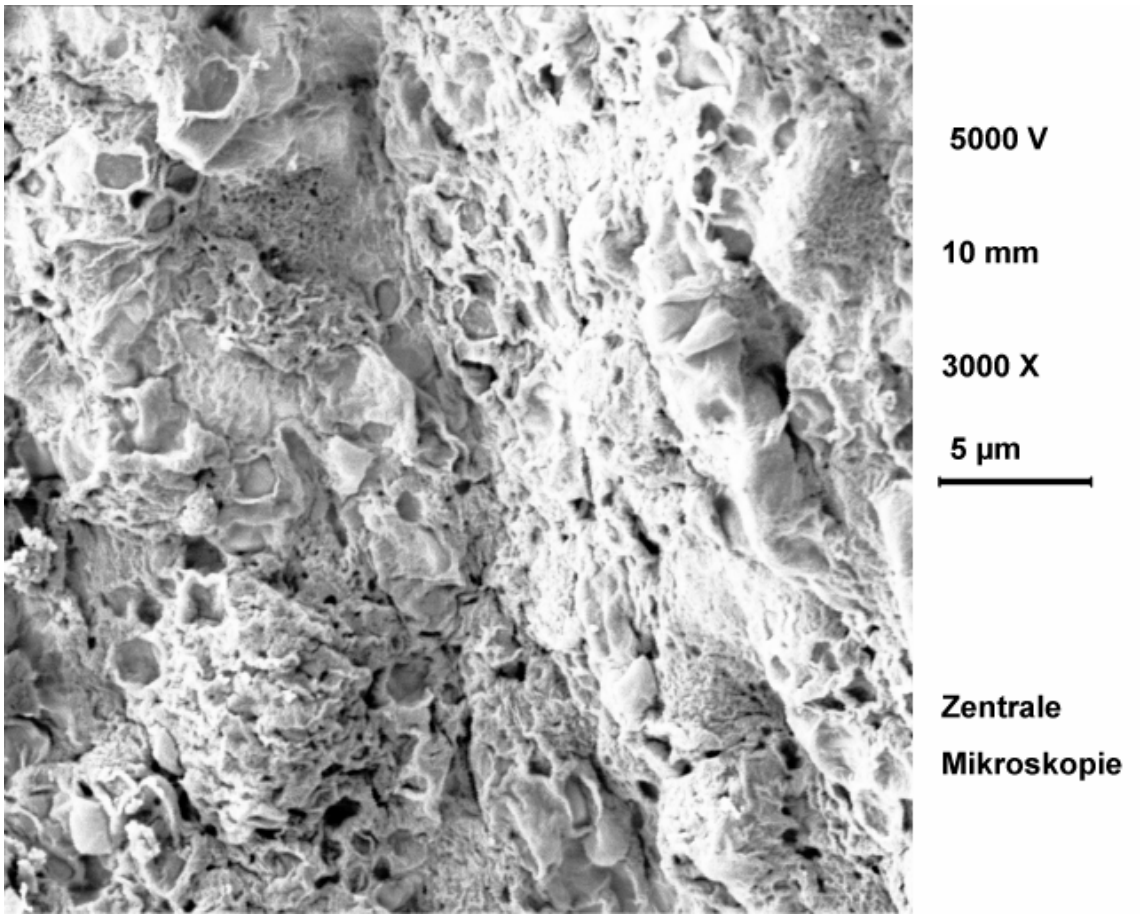


Fig. 4.14: M. leidyi after SEM-fixation. View on the surface with 3000x optical enlargement. The epithelium is disintegrated and the mesogloea can be seen.

4.3.6 Method 6 - Shock freezing

In a short pre-test for finding an adequate fixation method some healthy living individuals of *M. leidyi* were placed on a glass slide and than frozen at −20 °C over night. The results had been disappointing because the resigning water from the animal during the freezing process covered the surface and nothing more but ice crystals could have been observed. It was tried in various ways to remove the covering ice layer, e.g. scraping, carefully melting of the water but not the animal itself, and sublimation. All of them lead to a more or less intensive rupture and/or destruction of the comb jelly's surface.

In a next step small individuals were chosen and directly transfered into liquid nitrogen (−196 °C) for shock-freezing. After 5 minutes the animals were transfered into a Falcon Tube and stored at −70 °C until further processing. These animals were cut with a razorblade in smaller pieces and fixed to glass slides. They were studied under a cryo-electron-microscope (Hitachi S-4800 with a cryo apparatus from Gatan). The surface was sputtered with a 10nm layer of gold-palladium alloy. The epithelium is covered with ice and mucus which both prevent its direct observation but after a short time of sublimation the results were quite promising. Although the surface epithelium of *Mnemiopsis leidyi* is showing deletions again, the surface can be observed quite well (figures 4.15, 4.16, 4.17, and 4.18). But this method will need further adjustments. The major improvements include the removing of the mucus layer as far as possible, to allow observations of the epithelium directly and to compare epithelial and mucus conditions. Secondly, removing as much as possible of the attached water (e.g. avoid the Leidenfrost effect during the freezing), and stabilizing of the surface against sublimation damages have to be achieved.

The deletions of the epithelium increased in number and size during the observation period because of sublimation of the water. This provides good insights in the different layers of the mesogloea.

The aim of this study, getting a good picture of the undisturbed surface, is not completely reached so far but the method presented here gives promising results.

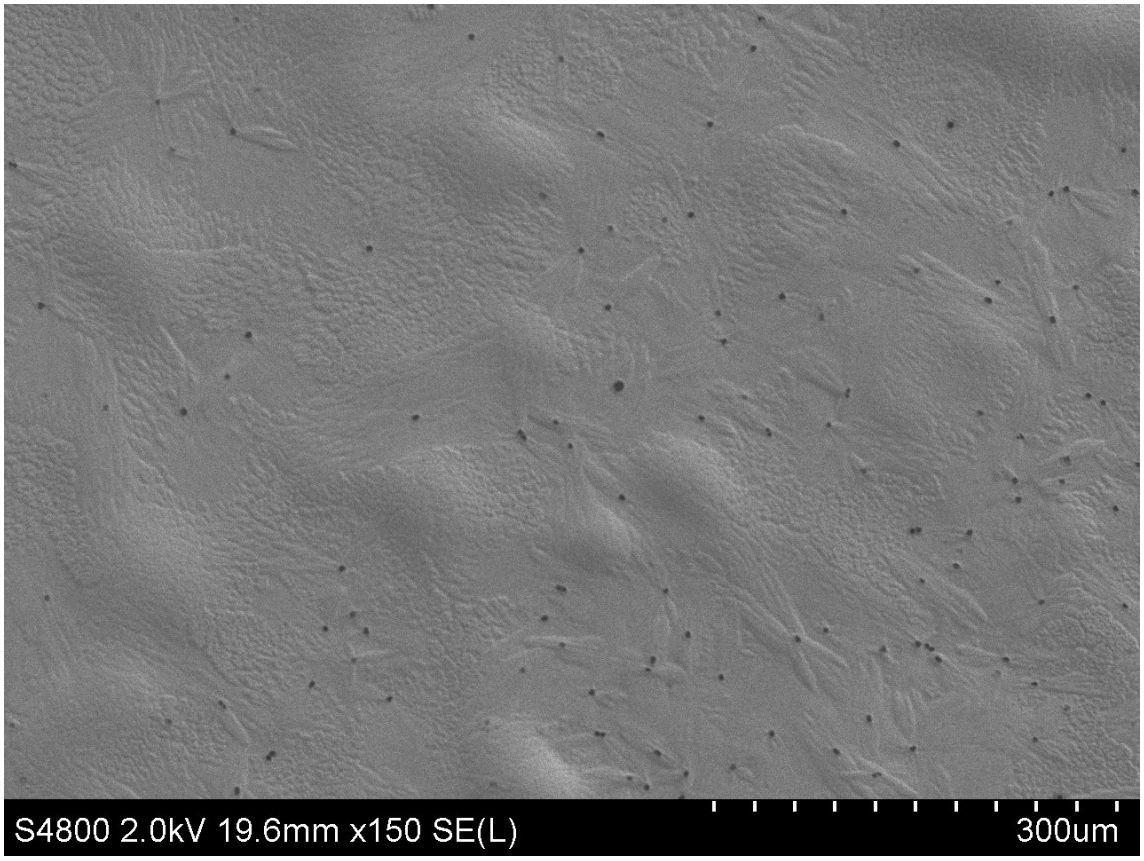


Fig. 4.15: Surface of *M. leidyi* after shock freezing in liquid nitrogen (−196 °C) with a cryo-electron-microscope, 150x optical enlargement. The black spots are ruptures of the surface by the sublimation processes. The surface is covered with the mucus layer of the animal but some structures are visible.

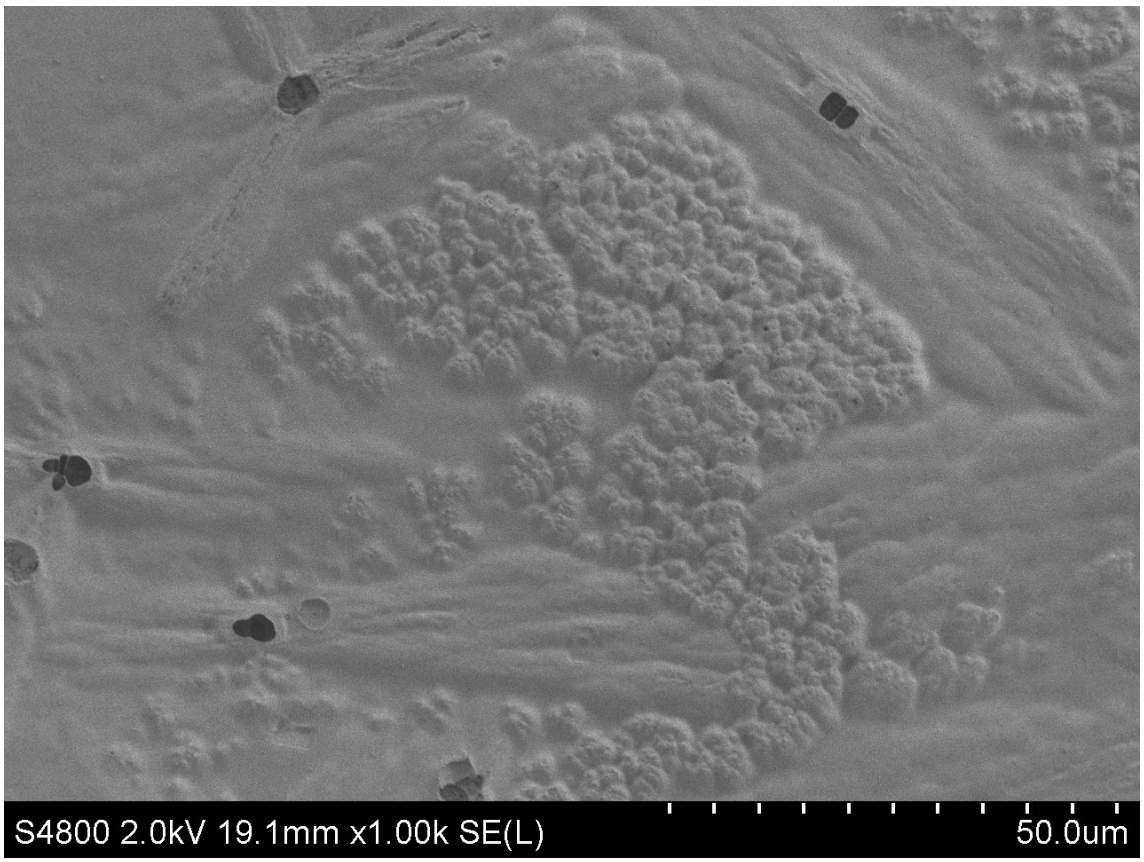


Fig. 4.16: Surface of *M. leidyi* after shock freezing in liquid nitrogen (−196 °C) with a cryo-electron-microscope, 1000x optical enlargement. The black spots from figure 4.15 can easily be identified now as ruptures of the surface. The cloudy structures show small pores, which indicates them to be a real surface structure and not artifacts.

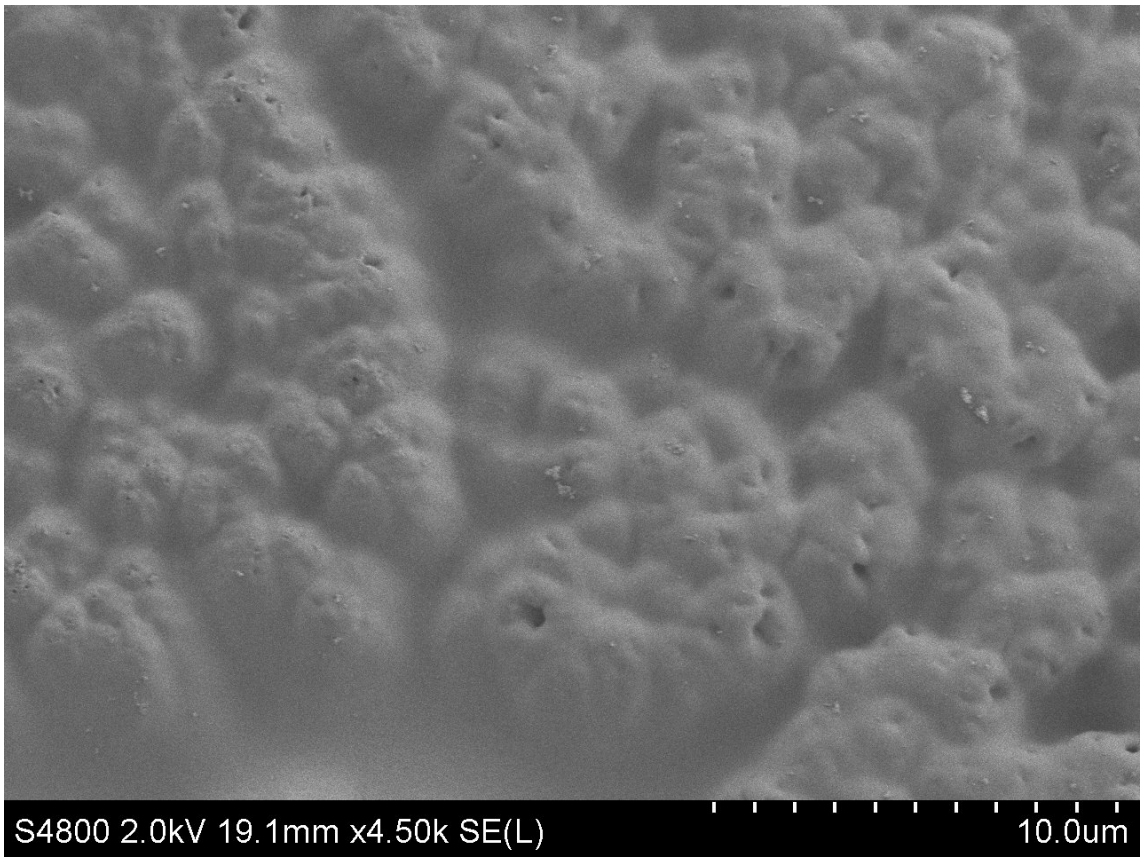


Fig. 4.17: Surface of *M. leidyi* after shock freezing in liquid nitrogen (−196 °C) with a cryo-electron-microscope, 4500x optical enlargement. A strong zoom into one of the cloudy structures from 4.16. The black spots are pores or channels of or through the epithelium.

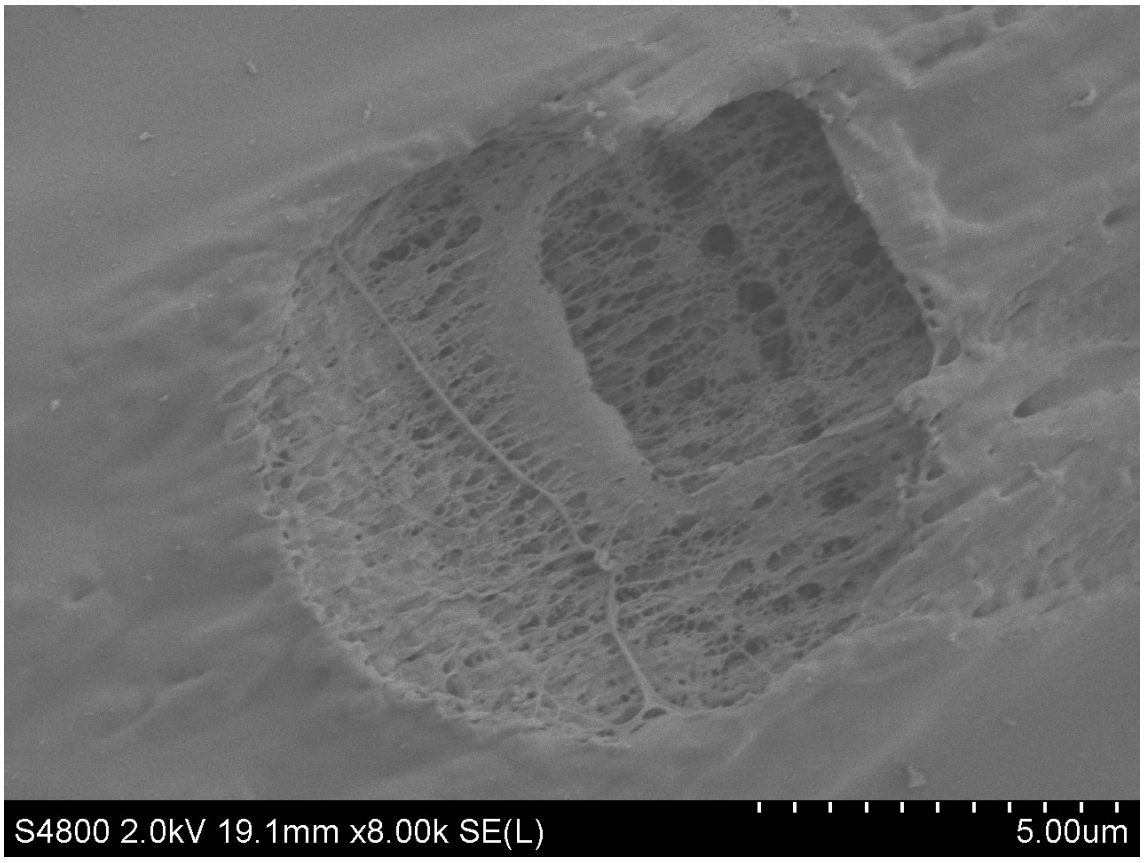


Fig. 4.18: Surface of *M. leidyi* after shock freezing in liquid nitrogen (−196 °C) with a cryo-electron-microscope, 8000x optical enlargement. The rupture is giving insight into the mesogloea and indicates an arrangement in layers; at least five different layers are visible here.

4.4 Discussion

The goal aimed for, developing a method to visualize the surface and its structures of *Mnemiopsis leidyi* in an undestroyed and undisturbed manner, was clearly not met in the first five studies presented here. Two major reasons can be hold responsible for that. On the one hand the animal itself is extremely fragile, physically as well as chemically. The methods presented in chapters 4.3.3 and 4.3.4 both lead to the total disappearance of the whole animal. Maybe it is possible to avoid such effects by adapting the liquids in these approaches in osmolarity/salinity to those of the animals. These modifications could not have been tested so far because a second problem arose. The stable culture of *Mnemiopsis leidyi* went extinct for unknown reasons after the movement from the GEOMAR - Helmholtz Centre of Ocean Research, Kiel to the Biological Center of the University of Kiel, where the tests were carried out. In addition, the bloom of the ctenophore population in the Baltic Sea and the North Sea in the autumn of the year 2011 failed for unknown reasons. Until now, a new laboratory culture could not have been established.

The most promising method tested so far is method 6, presented in chapter 4.3.6, the "shock-freezing". And, to a somewhat lesser extent methods 2 (chapter 4.3.2), "Drying", and 5 (chapter 4.3.5), "Fixation protocol for electron microscopy". For all three methods further tests are necessary, which means more animals will be needed. The AnoDisc approach 4.3.2 is promising if it is possible to get rid of the attached water and the resulting salt crystals after the drying process. The approach presented in chapter 4.3.5 might work if it would somehow be possible to avoid the damages arising during the dehydration process. Maybe the animal can be stabilized with starch solution, N,N-dimethylformamide (DMF) or another chemical (maybe cryo-protection-) solution, for potentially useful ideas see: Heeger et al. (1992); Meissner and Schwarz (1990); Yuan et al. (2008).

For the moment the shock-freezing method (in chapter 4.3.6) led to the best results of all of the tested approaches. Again the attached water and the mucus are the biggest problems to solve. To get rid of the mucus a method can be found in Garland et al. (1982) but it will be necessary to adapt it to ctenophores. The possibility to sublimate at least some of the attached water directly in the cryo-unit of the electron microscope is a great benefit of the used microscope (Hitachi S-4800 with a cryo apparatus from Gatan). The resulting pictures after two steps of sublimation are of much higher quality than before. This can be seen in figures 4.15 to 4.18.

So one of our basic questions from the introduction of this chapter can be answered at least in parts: Yes, there are really no bacteria nor archaea on the surface of *Mnemiopsis leidyi* detectable optically, at least not on the mucus. For now the

second question ("How does the comb jelly *M. leidyi* keep its surface not just clean but sterile?") is still lacking an answer. But at in the future the shock-freezing method might lead to pictures of adequate quality to answer this point, too. So far the pictures give no hint for a surface structure leading to the absence of a biofilm. Maybe the observed pores (see figure 4.17) secrete biofilm avoiding substances? The latter would suggest a (bio-)chemical mechanism - not a physical one - as the reason for the observed findings. But to be sure about that more pictures in high enlargements and maybe manipulation experiments will be necessary, and of course further chemical and genetical support will be needed.

It is planned to continue this way as soon as there are fresh animals available in the Kiel Bight. So it will hopefully be possible to establish some - or at least one - methods to learn more about the surface structures and quality characteristics of one of the oldest multicellular organism group in our oceans (Dunn et al., 2008; Hejnol et al., 2009). And furthermore we will understand more about the establishment or avoidance of biofilms on living organisms and the resulting effects on and of symbiotic interactions.

5. CONCLUDING DISCUSSION & OUTLOOK

"Cnidaria are somehow an alternative way of being a comb jelly"

(the author)

5.1 Concluding Discussion

The monitoring presented in chapter 2 indicates a stable and self-reproducing population of *Mnemiopsis leidyi* in the western Baltic Sea. This is in good agreement with the results of Lehmann and Javidpour (2010), who modeled the potential pathways of invasion and dispersal of the sea walnut in the Baltic Sea and excluded a yearly advection from the Kattegat. Furthermore it was shown that - at least for the moment - *M. leidyi* should be regarded as an alien but not an invasive species, as no negative effects of its presence were observed so far. Its main bloom in all monitored areas takes place later in the year than that of *Aurelia aurita* and no replacement of native makrozooplankton species was monitored up to now. In addition, Jaspers et al. (2011) showed that the effects on the eggs and therefore the reproduction rates of the Baltic cod (*Gadus morhua callarias* L.) are negligible even if both appear in the same time and area (Bornholm Basin) because of a lacking attraction of cod eggs as nutrition for the sea walnut. How these findings persist or change in the light of climate change cannot be predicted. With its high tolerance against abiotic factors like temperature and salinity (Purcell et al., 2001) and its omnivoric feeding habit it seems likely that the comb jelly will remain as new member in the Baltic Sea species community. But even small changes in temperature and salinity might change its interactions to the other species of the system - namely copepods, the moon jellyfish and other jellies and fishes like the cod - to the disadvantage of the latter. On the other hand, Moss et al. (2001a) noted that low salinities promote the infestation of the comb jelly by protists which might affect the performance of *M. leidyi*. Further studies will be needed to solve these questions.

In chapter 3, the investigation of symbiotic interactions of *M. leidyi* with prokaryotes revealed two interesting points. Firstly, the sea walnut *Mnemiopsis leidyi* shelters a small number of bacterial species in the inner parts of its body. These bacterial community is reducing during individual development from 20 ($\pm$ 11) bacterial phylotypes in larvae to 8 ($\pm$ 3) phylotypes in adult animals. Not only the pure number of bacterial phylotypes is reducing but also on a higher taxonomic level changes are taking place. The larvae of *M. leidyi* harbor bacteria from the classes γ-, β-, and ϵ-Proteobacteria as well as Bacteriodetes, Firmicutes, and Tenericutes. In contrast, the adult animals only harbor bacteria out of the classes α- and γ-Proteobacteria. Our findings correspond in parts with those of Daniels and Breitbart (2012) who found the same bacterial lineages as symbionts with *M. leidyi* when sampling in Tampa Bay, Florida, USA throughout the course of the year and analyzing the respective specimen. They did not distinguish between adult and larval specimen but could show a variation of the bacterial community in whole-body extracts over the sampling time. Unfortunately, it cannot be decided whether the variation in their

findings is due to the individual developmental stage or only to different sampling dates. In our case, it has to be emphasized that none of the detected bacterial phylotypes is found in both developmental stages. Why the bacterial community is changing is not clear. It might be possible that this finding is a side-effect of a changing diet throughout growing up. Another explanation might be that the immune system of *Mnemiopsis* is developing during individual growing. A combination of both effects seems to be most likely. Secondly, the surface epithelium of *M. leidyi* was found to be totally void of bacteria, despite the fact that symbionts are present in whole-body extracts. To the best of our knowledge this is the first time such an observation was made.

To investigate the astonishing finding of a sterile surface further, most suitably by screening the surface by electron microscopy for symbionts, a method to fixate specimen of *M. leidyi* with an undestroyed surface was searched for in chapter 4. Standard methods (examples can be found in Tamm and Tamm (1981) and Mulisch and Welsch (2010)) expose the samples to rather harsh conditions which lead in almost all cases to ruptures or even the complete destruction of the extremely sensitive epithelium of the sea walnut. Albeit some of these methods are known to work well for jellyfish fixation, they turned out to be not suitable for ctenophores. However, the "shock freezing" of *M. leidyi* specimen led to acceptable results (method 4.3.6). Although further adjustments would be needed to get excellent pictures, the preliminary images are on the whole satisfying and clearly support the finding mentioned before: Neither bacteria nor archaea can be found on the surface epithelium of *M. leidyi*. So far it was not possible to detect an underlying mechanism for this amazing observation. Unfortunately our culture of *Mnemiopsis leidyi* died during a movement of laboratories and the bloom in autumn 2011 failed - so no further tests could be conducted up to now. Nevertheless, if there is a structural surface mechanism to avoid bacterial settlement on the surface epithelium, the method of "shock freezing" is the most promising one to detect it. Such a structural effect could be an equivalent to the so-called lotus-effect (Barthlott and Neinhuis (1997)) on a bacterial scale. On the other hand, if there is a (bio-)chemical reason for the lack of symbionts, other approaches have to be carried out. In a first chemical screening neither polar (methanol) nor apolar (hexane) extracts exhibited any antibacterial molecules, which may prevent settlement or growth of bacteria (M. Wahl & M. Zimmer, unpubl.). Further tests with High Pressure Liquid Chromatography (HPLC) are planned.

Some of our initial questions could not be answered completely. Because of lacking appropriate samples a comparison of the host-symbiont communities of native and invaded habitats could not be performed up to now but is planned for the near future. The postulated idea of a "space-for-time" approach should be incorporated

in further investigations on host-symbiont interactions in changing environments, be them changed by an invasion process into a new habitat or by abiotic factors like a climate change. Instead of designing complex long term experiments with different environmental conditions - which is quite complicated for larger organisms - the invasion process itself sets the experimental design. The problem with an organism like the sea walnut is the lack of methods to investigate such questions. Although *Mnemiopsis leidyi* is in some fields an established model organism (developmental biology, taxonomy) it is not well suited for electron microscopic surface inspections. This thesis is laying a foundation to overcome this obstacle, so that the screening of *M. leidyi* for symbionts may become a regularly used indicator for its adaption to a new or changing environment. The final intention of this is to establish the sea walnut as a model organism for the aforementioned "space-for-time" approach. Because *Mnemiopsis leidyi* fulfills some important requirements as to act as a routinely investigated model organism - like easy-to-catch, high abundances and long-term observation in many areas - it seems promising to continue in this direction. Particularly because of the great differences in environmental conditions of native and invaded habitats (US east coast, the Baltic, and the Black Sea), a thorough comparison of specimen originating from all this different areas is likely to reveal crucial factors for a successful invasion or rather the ability to survive climate changes.

Various authors like Fraune (2008) and Zilber-Rosenberg and Rosenberg (2008) pointed out that interaction with the environment is a complicated network of interactions between the host, its parasites, commensales, mutualists, prey, predators and the environment itself (figure 5.1).

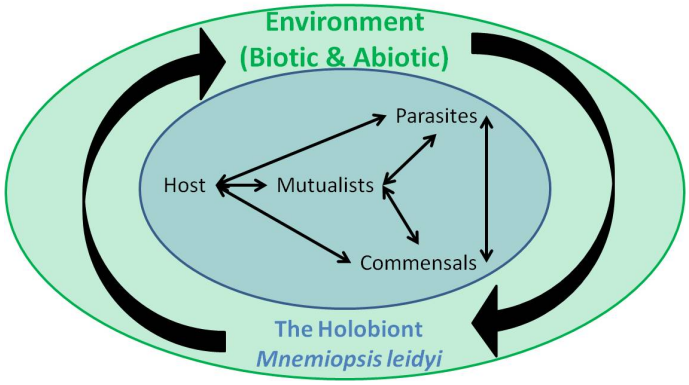


Fig. 5.1: The Holobiont in its environment. The interactions between all members of the holobiont, e.g. the host, parasites, commensales, and mutualists, and the holobionts interactions with the environment are shown schematically.

As mentioned above, an exceptional finding in this thesis is the absence of protists correlated with *M. leidyi* in the Baltic, although it has to be noted that such interactions have been described by various authors for other regions of the world (Mills and McLean, 1991; Estes et al., 1997; Moss et al., 2001b). It seems likely that their loss is closely linked to the conditions of the new habitat, despite the described higher infestation rates of the sea walnut with protists at lower salinities (Moss et al., 2001a). Up to now it cannot be said if this is an advantage or a drawback for *Mnemiopsis leidyi*. A personal observation illustrates this puzzle: In the Baltic, a relatively high number of dead *M. leidyi* larvae has been spotted, densely covered by bacteria or fungi. As such a finding has not been reported from other sampling areas, a possible reason might be the absence of the protists which could confine bacteria and fungi by feeding on them.

Worth thinking about is also the question if only *M. leidyi* specimen with a sterile surface (due to structural or biochemical effects) are capable of invading a new habitat as different from their natural one as the Baltic - or if a specimen normally inhabited by bacteria and/or protists looses them all when entering the new environment. In this context, laboratory experiments on *M. leidyi* cultures catched in different habitats, exposing them to their own and varying environmental conditions, might be useful. Also genetic variations as a function of habitat should be checked in further studies.

5.2 Outlook

As a first step, the improvement of the most promising fixation method for the SEM measurements (shock-freezing in liquid nitrogen) will go on. Based on that, the optical and physical characterization of the surface of the sea walnut will be completed. Maybe the absence of bacteria, if these results persist, is based on structural surface characteristics somehow comparable to the lotus-effect (Barthlott and Neinhuis, 1997) on bacterial size scales. Another explanation for the lack of bacteria on the surface might be a chemical/biochemical effect by e.g. antimicrobial peptides or any other antibiotic molecules, possibly combined with a structural effect. To check for such a (bio-)chemical factor, a chemical analyses of comb jelly whole-body extracts via high-performance liquid chromatography is planned. Material for this purpose is already collected and in parts prepared.

If the underlying mechanisms to avoid bacterial settlement - or at least to control it - are understood, manipulation experiments can be started to answer the following (and more) questions:

- Is the sterile surface a key to the invasive success of *M. leidyi*?
- How does the situation change under stress conditions (for host and/or potential symbionts)?
- Are symbiotic interactions prone to environmental conditions?
- Is individual and evolutionary development of *Mnemiopsis leidyi* and ctenophores as a whole influenced by symbionts?
- Can a sterile surface in sea water be mimicked, to circumvent the ever-present problem of biofilm formation on scientific or naval equipment and ships?

In addition, is still unknown whether the bacteria-free surface is a general feature of *Mnemiopsis leidyi* or whether it is an artifact somehow correlated to the invasion process itself: the symbiotic organisms might not be able to face the new conditions while the comb jellies are. To elucidate this matter, surface samples of *M. leidyi* were collected in its native range, the U.S. East Coast. These sampled are stored in 75% ethanol and will be sequenced and analyzed in the upcoming months, depending on fundings.

Finally it is still not clear whether the invasions of the Baltic Sea and the Northern Sea by the sea walnut are completed, nor if they are successful at all. This is the topic of current projects, like those of Jamileh Javidpour at the Leibniz-Institute of Marine Sciences, IFM-GEOMAR in Kiel.

6. APPENDIX

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation nach den Regeln guter wissenschaftlicher Praxis selbst verfasst habe. Dabei habe ich keine Hilfe, außer der wissenschaftlichen Beratung durch meinen Doktorvater PD Dr. Martin Zimmer und meinen Zweitbetreuer Prof Dr. Philip Rosenstiel in Anspruch genommen.
Diese Arbeit hat weder ganz noch in Teilen einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegen. Teile dieser Arbeit wurden zur Veröffentlichung eingereicht.
Desweiteren erkläre ich hiermit, dass ich noch keinen Promotionsversuch unternommen habe.

Kiel, den 18.12.2012

Sven Hammann

Data

The data acquired in the course of this work can be found in pdf- and excel-files on the attached CD.

REFERENCES

Baker, L. and Reeve, M. (1974). Laboratory Culture of the Lobate Ctenophore Mnemiopsis mccradyi with Notes on Feeding and Fecundity. *Marine Biology*, 26:57–62.

Barthlott, W. and Neinhuis, C. (1997). Purity of the sacred lotus, or escape from contamination in biological surfaces. *Planta*, 202(1):1–8.

Bilio, M. and Niermann, U. (2004). Is the comb jelly really to blame for it all? Mnemiopsis leidyi and the ecological concerns about the Caspian Sea. *Marine Ecology Progress Series*, 269:173–183.

Blumenthal, D. M. (2006). Interactions between resource availability and enemy release in plant invasion. *Ecology letters*, 9(7):887–95.

Boersma, M., Malzahn, A. M., Greve, W., and Javidpour, J. (2007). The first occurrence of the ctenophore Mnemiopsis leidyi in the North Sea. *Helgoland Marine Research*, 61(2):153–155.

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scand J Statist*, 11:265–270.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., and Wagner, M. (1999). The Domain-specific Probe EUB388 is Insufficient for the Detection of all Bacteria: Development and Evaluation of a more Comprehensive Probe Set. *Sytematic and Apllied Microiology*, 22:434–444.

Dale, C. and Moran, N. A. (2006). Molecular interactions between bacterial symbionts and their hosts. *Cell*, 126(3):453–65.

Daniels, C. and Breitbart, M. (2012). Bacterial communities associated with the ctenophores Mnemiopsis leidyi and Beroe ovata. *FEMS microbiology ecology*, pages 1–12.

Deason, E. E. (1982). Mnemiopsis leidyi (Ctenophora) in Narragansett Bay, 1975-79: Abundance, Size Composition and Estimation of Grazing. *Estuarine, Coastal and Shelf Science*, 15:121–134.

DeWalt, S. J., Denslow, J. S., and Ickes, K. (2004). Natural-Enemy Release Facilities Habitat Expansion of the Invasive Tropical Shrub Clidemia hirta. *Ecology*, 85(2):471–483.

Dunn, C. W., Hejnol, A., Matus, D. Q., Pang, K., Browne, W. E., Smith, S. A., Seaver, E., Rouse, G. W., Obst, M., Edgecombe, G. D., Sø rensen, M. V., Haddock, S. H. D., Schmidt-Rhaesa, A., Okusu, A., Kristensen, R. M. b., Wheeler, W. C., Martindale, M. Q., and Giribet, G. (2008). Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature*, 452(7188):745–9.

Elton, C. S. (1958). *The ecology of invasions by animals and plants*. University of Chicago Press.

Estes, A. M., Reynolds, B. S., and Moss, A. G. (1997). Trichodina ctenophorii n. sp., a novel symbiont of ctenophores of the northern coast of the Gulf of Mexico. *The Journal of eukaryotic microbiology*, 44(5):420–6.

Faasse, M. A. and Bayha, K. M. (2006). The ctenophore Mnemiopsis leidyi A. Agassiz 1865 in coastal waters of the Netherlands: an unrecognized invasion? *Aquatic Invasions*, 1(4):270–277.

Fraune, S. (2008). *Towards understanding a holobiont: Host-microbe interactions in Hydra*. Dissertation, Christian-Albrechts-Universiät, Kiel.

Fraune, S. and Bosch, T. C. G. (2007). Long-term maintenance of species-specific bacterial microbiota in the basal metazoan Hydra. *Proceedings of the National Academy of Sciences of the United States of America*, 104(32):13146–51.

Fraune, S. and Zimmer, M. (2008). Host-specificity of environmentally transmitted Mycoplasma-like isopod symbionts. *Environmental Microbiology*, 10(10):2497–2504.

Fuentes, V., Atienza, D., Gili, J.-M., and Purcell, J. (2009). First records of Mnemiopsis leidyi A. Agassiz 1865 off the NW Mediterranean coast of Spain. *Aquatic Invasions*, 4(4):671–674.

Garland, C. D., Nash, G. V., and Mcmeekin, T. A. (1982). Absence of Surface-Associated Microorganisms in Adult Oysters (Crassostrea gigas). *Applied and Environmental Microbiology*, 44(5):1205–1211.

Gasca, R. and Haddock, S. H. D. (2004). Associations between gelatinous zooplankton and hyperiid amphipods (Crustacea: Peracarida) in the Gulf of California. *Hydrobiologia*, 530/531:529–535.

GESAMP (1997). Opportunistic settlers and the problem of M. leidyi invasion in the Black Sea. Technical report, GESAMP (IMO/FAO/UNESCO-IOC/WMO/WHO/IAEA/UNEP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection) reports and studies No. 58. International Maritime Organization, London.

Gucu, A. (2002). Can Overfishing be Responsible for the Successful Establishment of Mnemiopsis leidyi in the Black Sea? *Estuarine, Coastal and Shelf Science*, 54(3):439–451.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41:95–98.

Hansson, H. G. (2006). Ctenophores of the Baltic and adjacent Seas: the invader Mnemiopsis is here! *Aquatic Invasions*, 1(4):295–298.

Heeger, T., Piatkowski, U., and Moller, H. (1992). Predation on jellyfish by the cephalopod Argonauta argo. *Marine Ecology Progress Series*, 88:293–296.

Hejnol, A., Obst, M., Stamatakis, A., Ott, M., Rouse, G. W., Edgecombe, G. D., Martinez, P., Baguñà, J., Bailly, X., Jondelius, U., Wiens, M., Müller, W. E. G., Seaver, E., Wheeler, W. C., Martindale, M. Q., Giribet, G., and Dunn, C. W. (2009). Assessing the root of bilaterian animals with scalable phylogenomic methods. *Proceedings. Biological sciences/The Royal Society*, 276(1677):4261–70.

Henne, A., Daniel, R., Schmitz, R. A., and Gottschalk, G. (1999). Construction of Environmental DNA Libraries in Escherichia coli and Screening for the Presence of Genes Conferring Utilization of 4-Hydroxybutyrate. *Applied and Environmental Microbiology*, 65(9):3901–3907.

Henry, J. Q. and Martindale, M. Q. (2000). Regulation and regeneration in the ctenophore Mnemiopsis leidyi. *Developmental biology*, 227(2):720–33.

Isinibilir, M. and Tarkan, A. N. (2002). Distribution of the Invasive Ctenophore Mnemiopsis leidyi (Agassiz, 1865) in the North-eastern Aegean Sea in August 1998. *Turkish Journal of Fisheries and Aquatic Sciences*, 2:129–132.

Ivanov, V. P., Kamakin, A. M., Ushivtzev, V. B., Shiganova, T., Zhukova, O., Aladin, N., Wilson, S. I., Harbison, G. R., and Dumont, H. J. (2000). Invasion of the Caspian Sea by the comb jellyfish Mnemiopsis leidyi (Ctenophora). *Biological Invasions*, 2:255–258.

Jaspers, C., Titelman, J., Hansson, J., Haraldsson, M., and Rø llike, C. (2011). The invasive ctenophore Mnemiopsis leidyi poses no direct threat to Baltic cod eggs and larvae. *Limnology and Oceanography*, 56(2):431–439.

Javidpour, J. (2006). First record of Mnemiopsis leidyi A. Agassiz 1865 in the Baltic Sea. *Aquatic Invasions*, 1(4):299–302.

Javidpour, J., Molinero, J. C., Peschutter, J., and Sommer, U. (2008). Seasonal changes and population dynamics of the ctenophore Mnemiopsis leidyi after its first year of invasion in the Kiel Fjord, Western Baltic Sea. *Biological Invasions*, 11(4):873–882.

Jeffrey, S. and Humphrey, G. (1975). New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants algae and natural phytoplankton. *Biochem. Physiol. Pflanzen*, 167:191–194.

Keane, R. and Crawley, M. (2002). Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology and Evolution*, 17(4):164–170.

Khan, R., Berker, D., Williams-Ryan, K., and Hooper, R. (1993). Influence of crude oil and pulp and paper mill effluent on mixed infections of Trichodina cottidarium and T. saintjohnsi (Ciliphora) parasitizing Myoxiocephalus octodecemspinosus and M. scorpius. *Canadian Journal of Zoology*, 72:247–251.

Khan, R. A. (1990). Parasitism in marine fish after chronic exposure to petroleum hydrocarbons in the laboratory and to the Exxon Valdez oil spill. *Bulletin of environmental contamination and toxicology*, 44(5):759–63.

Kideys, A. E. (2002). Ecology. Fall and rise of the Black Sea ecosystem. *Science (New York, N.Y.)*, 297(5586):1482–4.

Kideys, A. E. and Niermann, U. (1994). Occurence of Mnemiopsis along the Turkish coast. *ICES Journal of Marine Science*, 51:423–427.

Kremer, P. (1994). Patterns of abundance for Mnemiopsis in US coastal waters: a comparative overview. *Journal of marine Sciences*, 51:347–354.

Lehmann, A. and Javidpour, J. (2010). Potential pathways of invasion and dispersal of Mnemiopsis leidyi A. Agassiz 1865 in the Baltic Sea. *Hydrobiologia*, 649(1):107–114.

Lockwood, J., Hoopes, M., and Marchetti, M. (2007). *Invasion ecology*. Wiley-Blackwell, 1. edition.

Lowe, S., Browne, M., Boudjelas, S., and De Porter, M. (2004). 100 of the World's Worst Invasive Alien Species. *The Invasive Species Specialist Group (ISSG) of the Species Survival Commission (SSC) of the World Conservation Union (IUCN)*, 12:12.

MacKenzie, B. R., Gislason, H., Mölmann, C., and Köster, F. W. (2007). Impact of 21st century climate change on the Baltic Sea fish community and fisheries. *Global Change Biology*, 13(7):1348–1367.

Margulis, L. (1993). *Symbiosis in Cell Evolution: Microbial Communities in the Archean and Proterozoic Eons*. W.H. Freeman & Co, 2 edition.

Martindale, M. Q. (1987). Larval reproduction in the ctenophore Mnemiopsis mccradyi (order Lobata). *Marine Biology*, 94:409–414.

Martorelli, S. R. (2001). Digenea parasites of jellyfish and ctenophores of the southern Atlantic. *Hydrobiologia*, 451:305–310.

Medina, M., Collins, a. G., Silberman, J. D., and Sogin, M. L. (2001). Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. *Proceedings of the National Academy of Sciences of the United States of America*, 98(17):9707–12.

Meissner, D. H. and Schwarz, H. (1990). Improved Cryoprotection and Freeze-Substitution of Embryonic Quail Retina: A TEM Study on Ultrastructural Preservation. *Journal of Electron Microscopy Technique*, 14:348–356.

Mills, C. and McLean, N. (1991). Ectoparasitism by a dinoflagellate (Dinoflagellata: Oodinidae) on 5 ctenophores (Ctenophora) and a hydromedusa (Cnidaria). *Diseases of Aquatic Organisms*, 10:211–216.

Mills, C. E. (1995). Medusae , siphonophores , and ctenophores as planktivorous predators in changing global ecosystems. *Journal of marine Sciences*, 52:575–581.

Mills, C. E. (1998). Phylum Ctenophora: A List of all Valid Species Names. Electronic internet document available at http://faculty.washington.edu/cemills/Ctenolist.html. Published by the author, web page established March 1998, last updated (see date at end of page).

Minelli, A. (2007). Invertebrate taxonomy and evolutionary developmental biology. *Zootaxa*, 1668:55 – 60.

Minelli, A. (2009). *Perspectives in Animal Phylogeny & Evolution*. Oxford University Press, New ork, 1 edition.

Minelli, A., Negrisolo, E., and Fusco, G. (2006). Reconstructing Animal Phylogeny in the Light of Evolutionary Developmental Biology. In Hodkinson, T. R. and Pamel, J. A. n., editors, *Taxonomy and Systematics of species Rich Taxa*, chapter 12, pages 177–190. CRC Press.

Mitchell, C. E. and Power, A. G. (2003). Release of invasive plants from fungal and viral pathogens. *Nature*, 421(6923):625–7.

Moran, N. a. and Baumann, P. (2000). Bacterial endosymbionts in animals. *Current opinion in microbiology*, 3(3):270–5.

Moss, A. G., Estes, A. M., Muellner, L. A., and Morgan, D. D. (2001a). Protistan epibionts of the ctenophore Mnemiopsis mccradyi Mayer. *Hydrobiologia*, 451:295–304.

Moss, A. G., Rapoza, R. C., and Muellner, L. (2001b). A novel cilia-based feature within the food grooves of the ctenophore Mnemiopsis mccradyi Mayer. *Hydrobiologia*, 451:287–294.

Mulisch, M. and Welsch, U. (2010). *Romeis Mikroskopische Technik*. Spektrum Akademischer Verlag, Heidelberg, 18 edition.

Neumann, T. (2010). Climate-change effects on the Baltic Sea ecosystem: A model study. *Journal of Marine Systems*, 81(3):213–224.

Pang, K. and Martindale, M. Q. (2008). Quick guide Ctenophores. *Current Biology*, pages 1119–1120.

Podar, M., Haddock, S. H., Sogin, M. L., and Harbison, G. R. (2001). A molecular phylogenetic framework for the phylum Ctenophora using 18S rRNA genes. *Molecular Phylogenetics and Evolution*, 21(2):218–230.

Purcell, J. E., Shiganova, T. A., Decker, M. B., and Houde, E. D. (2001). The ctenophore Mnemiopsis in native and exotic habitats : U . S . estuaries versus the Black Sea basin. *Hydrobiologia*, 451:145–176.

RCoreTeam (2012). R: A Language and Environment for Statistical Computing.

Reitzel, A. M., Sullivan, J. C., Brown, B. K., Chin, D. W., Cira, E. K., Edquist, S. K., Genco, B. M., Joseph, O. C., Kaufman, C. A., Kovitvongsa, K., Muñoz, M. M., Negri, T. L., Taffel, J. R., Zuehlke, R. T., and Finnerty, J. R. (2007).

Ecological and developmental dynamics of a host-parasite system involving a sea anemone and two ctenophores. *The Journal of parasitology*, 93(6):1392–402.

Reusch, T. B. H., Bolte, S., Sparwel, M., Moss, A. G., and Javidpour, J. (2010). Microsatellites reveal origin and genetic diversity of Eurasian invasions by one of the world's most notorious marine invader, Mnemiopsis leidyi (Ctenophora). *Molecular ecology*, 19(13):2690–9.

Schroedter, L. (2008). *Mnemiopsis leidyi: Aquakultur und Reproduktionserfolg unter verschiedenen abiotischen Faktoren*. Besondere lernleistung, Christian-Albrechts-Universität, Kiel.

Shiganova, T. (1993). Ctenophore Mnemiopsis leidyi and ichthyplankton in the Sea of Marmara in October of 1992. *Oceanology*, 33:900–903.

Shiganova, T. (2000). Effects of gelatinous plankton on Black Sea and Sea of Azov fish and their food resources. *ICES Journal of Marine Science*, 57(3):641–648.

Shiganova, T. A. (1998). Invasion of the Black Sea by the ctenophore Mnemiopsis leidyi and recent changes in pelagic community structure. *Fisheries Oceanography*, 7(3-4):305–310.

Shiganova, T. A., Musaeva, E. I., Bulgakova, Y. V., Mirzoyan, Z. A., and Martynyuk, M. L. (2003). Invaders Ctenophores Mnemiopsis leidyi (A . Agassiz) and Beroe ovata Mayer 1912, and Their Influence on the Pelagic Ecosystem of Northeastern Black Sea. *Biology Bulletin*, 30(2):180–190.

Steinert, M., Hentschel, U., and Hacker, J. (2000). Symbiosis and pathogenesis: evolution of the microbe-host interaction. *Die Naturwissenschaften*, 87(1):1–11.

Studenikina, Y., Volovik, S., Mirzoyan, I., and Luts, G. (1991). The ctenophore Mnemiopsis leidyi in the Sea of Azov. *Oceanology*, 31:722–725.

Sullivan, B. K., Keuren, D. V., and Clancy, M. (2001). Timing and size of blooms of the ctenophore Mnemiopsis leidyi in relation to temperature in Narragansett Bay, RI. *Hydrobiologia*, 451:113–120.

Sullivan, L. J. and Gifford, D. J. (2007). Growth and feeding rates of the newly hatched larval ctenophore Mnemiopsis leidyi A. Agassiz (Ctenophora, Lobata). *Journal of Plankton Research*, 29(11):949–965.

Tamm, S. L. and Tamm, S. (1981). Ciliary reversal without rotation of axonemal structures in ctenophore comb plates. *The Journal of cell biology*, 89(3):495–509.

Vinogradov, M., Shushkina, E., Musayeva, E., and Sorokin, P. (1989). A new exotic species in the Black Sea: the ctenophore Mnemiopsis leidyi (Ctenophore: Lobata). *Oceanology*, 29:220–224.

Wallberg, A., Thollesson, M., Farris, J. S., and Jondelius, U. (2004). The phylogenetic position of the comb jellies (Ctenophora) and the importance of taxonomic sampling. *Cladistics*, 20(6):558–578.

Weisburg, W. G., Barns, S. M., Pelletier, D. a., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*, 173(2):697–703.

Williamson, M. and Fitter, A. (1996). The Varying Success of Invaders. *Ecology*, 77(6):1661.

Yuan, D., Nakanishi, N., Jacobs, D. K., and Hartenstein, V. (2008). Embryonic development and metamorphosis of the scyphozoan Aurelia. *Development genes and evolution*, 218(10):525–39.

Zaiko, A., Lehtiniemi, M., Narščius, A., and Olenin, S. (2010). Assessment of bioinvasion impacts on a regional scale: a comparative approach. *Biological Invasions*, 13(8):1739–1765.

Zilber-Rosenberg, I. and Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS microbiology reviews*, 32(5):723–35.