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Population genetics and ecological investigations of the common shipworm *Teredo navalis* Linnaeus, 1758 in European waters

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Summary

The widespread common shipworm *Teredo navalis* L., 1758 is a marine wood-boring bivalve and causes immense economic damages through the infestation of natural (e.g. mangrove roots, drift-wood) and anthropogenic wood sources (e.g. groyne piles, harbor facilities). The destruction of coastal protection structures and underwater cultural heritage results in substantial consequential costs and requires reliable species identification for statements about the current distribution area. First scientific reports on *T. navalis* for Central Europe date back to 1731 for the North Sea and to 1835 for the Baltic Sea, respectively. In the following decades, several sporadic mass occurrences have been stated for both areas. Since the end of the 20th century, a permanent, self-reproducing population has been monitored in the Baltic Sea. In this thesis, 430 specimens of Teredinidae from sampling sites in Europe and North America were investigated using molecular taxonomy. Several molecular markers, two nuclear (18S/28S) as well as one mitochondrial marker (cytochrome-c-oxidase subunit I, hereafter COI) were used. For the first time, a newly developed specific primer pair for *T. navalis* allowed PCR and sequencing of a 675 bp COI gene fragment and species identification via the DNA barcoding approach. Thus, it could be scientifically proven that all specimens belong to only one species and no sibling species of *T. navalis* exist in the investigated area. The classification into the system of wood-boring bivalves by using the nuclear dataset for a phylogenetic tree calculation revealed no differentiation between specimens of *T. navalis* from Europe and North America.

The mtDNA analyses showed high COI haplotype diversities in combination with low nucleotide diversities and a star-like network phylogeny with a predominant central haplotype. Various common population genetic indices, e.g. fixation index (F_{ST}) and an AMOVA analysis were calculated to reveal the present population structure. Finally, these calculations could not determine differentiated populations or any kind of different demes or lineages. Therefore, the results of this thesis point to a regional panmictic population in Central Europe reflected by a high gene flow with unhindered migration of individuals (e.g. via pelagic larvae). In addition, the past demographic structure of *T. navalis* was analyzed. The calculated values of Tajima's D, Fu's F and the mismatch distribution indicate a recent sudden population expansion but no signs of either a bottleneck or a founder effect.

To determine whether a range expansion of *T. navalis* in the Baltic Sea is observable, the larval settlement has been monitored over a period of four years. Wooden test panels were deployed along the prevailing salinity gradient and temperature and salinity were recorded in the adjacent water. During the investigation period, strong variations of the borehole abundances were found. However, no correlation was found between the key factors temperature and salinity and the borehole abundances. Analogous to previous studies, no current range expansion of *T. navalis* towards the east and thus areas of the Baltic Sea with lower salinities could be detected.

Zusammenfassung

Die marine Schiffsbohrmuschel *Teredo navalis* L., 1758 (Mollusca: Bivalvia: Teredinidae) ist weltweit verbreitet und verursacht durch den Befall natürlicher (z.B. Mangrovenwurzeln, Treibholz) sowie anthropogener Holzquellen (z.B. Buhnenpfähle, Hafenanlagen) immense ökonomische Schäden. Die Zerstörung von Küstenschutzbauwerken und des Unterwasserkulturerbes kann zu erheblichen Folgekosten führen und erfordert für Aussagen zum aktuellen Verbreitungsgebiet eine zuverlässige Artidentifikation.

Erste Berichte über *T. navalis* in Mitteleuropa stammen aus den Jahren 1731 für die Nordsee und 1835 für die Ostsee. In den folgenden Jahrzehnten wurden für beide Gebiete mehrere unregelmäßig auftretende Massenvorkommen beschrieben und erst seit Ende des 20. Jahrhunderts ist in der Ostsee eine permanente, sich selbst reproduzierende Population bekannt. In dieser Arbeit wurden insgesamt 430 Exemplare der Teredinidae aus Europa und Nordamerika molekular-taxonomisch untersucht. Es wurden zwei Kernmarker (18S/28S) sowie ein mitochondrialer Marker (Cytochrom-c-Oxidase Untereinheit I, nachfolgend COI) verwendet. Neu entwickelte, spezifische Primer für *T. navalis* erlaubten zum ersten Mal die Vervielfältigung und Sequenzierung eines 675 bp langen COI Genfragmentes und die Artbestimmung mittels ‚DNA barcoding'. Es konnte festgestellt werden, dass alle untersuchten Exemplare aus Nordsee und Ostsee zur Art *T. navalis* gehören und im Untersuchungsgebiet keine Geschwisterarten existieren. Der 18S/28S Datensatz wurde für eine phylogenetische Stammbaumberechnung verwendet und zeigte keine Differenzierung zwischen Individuen von *T. navalis* aus Europa und Nordamerika.

Die mtDNA-Analysen zeigten eine große genetische Diversität und eine sternförmige Haplotypennetzwerk-Phylogenie mit einem dominierenden zentralen Haplotyp. Verschiedene populationsgenetische Indizes wie z.B. der Fixierungsindex (F_{ST}) und eine AMOVA-Analyse wurden berechnet, konnten aber keine differenzierten Populationen aufzeigen. Die Ergebnisse deuten vielmehr auf eine regionale panmiktische Population in Mitteleuropa hin, die durch einen hohen Genfluss mit ungehinderter Migration von Individuen (z.B. über pelagische Larven) charakterisiert ist. Darüber hinaus wurde die historische demographische Struktur von *T. navalis* analysiert. Die berechneten Werte von Tajima's D und Fu's F sowie eine Mismatch-Analyse deuten auf eine plötzliche Populationsexpansion hin. Dabei konnten keine Anzeichen für einen Flaschenhals- oder Gründereffekt ermittelt werden.

Um festzustellen, ob eine Ausbreitung von *T. navalis* in der Ostsee in Richtung Osten beobachtet werden kann, wurden die Larvenansiedlungen über einen Zeitraum von vier Jahren beobachtet. Entlang des vorherrschenden Salzgehaltsgradienten wurden Testhölzer im Wasser exponiert, während gleichzeitig Wassertemperatur und Salzgehalt erfasst wurden. Im Untersuchungszeitraum wurden starke Schwankungen der Bohrlochabundanzen, jedoch keine Korrelation zwischen den Schlüsselfaktoren Temperatur und Salzgehalt und der Bohrlochanzahl als Maß des Larvenfalls ermittelt. Analog zu früheren Studien konnte keine Ausdehnung des Verbreitungsgebietes von *T. navalis* nach Osten und damit in Gebiete der Ostsee mit geringerem Salzgehalt nachgewiesen werden.

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List of abbreviations

°C	Degrees Celsius
18S	Nuclear 18S rRNA gene
28S	Nuclear 28S rRNA gene
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
BOLD	Barcode of Life Data
bp	Base pair
CA	California
COI	Cytochrome-c-oxidase subunit I gene
cm	Centimeter
cm^2	Square centimeter
dm^2	Square decimeter
DNA	Deoxyribonucleic acid
DIN	German industry standard
dNTP	2´-Desoxyribonucleosid-5´-triphosphate
DS 1/2	Dataset one and two
FL	Florida
F_{ST}	Fixation index
GPS	Global Positioning System
H	Number of haplotypes
Hd	Haplotype diversity
K	Number of nucleotide differences
K2P	Kimura 2-parameter model
km	Kilometer
µl	Microliter
MBI	Major Baltic Inflow
$MgCl_2$	Magnesium chloride
m	Meter
ML	Maximum likelihood
mM	Millimolar
µm	Micrometer
MS	Mediterranean Sea
mtDNA	Mitochondrial DNA

Mya	Million years ago
µM	Micro molar
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
pH	Potential of hydrogen
pmol	Picomol
π	Nucleotide diversity
rRNA	Ribosomal ribonucleic acid
SSD	Sum of square deviations
StALU MM	State Agency for Agriculture and Environment of Central Mecklenburg
τ	Tau
U	Unit
US	North American East Coast
UV	Ultraviolet

1 Introduction

1.1 Bivalve wood-boring mollusks

Marine wood-boring organisms are of various shapes and originate from different groups such as Crustacea or Bivalvia. The Teredinidae (commonly known as shipworms) are distributed worldwide from the polar to the tropical zones probably representing the group of the most wood-destructive and cost-incurring marine invertebrates (Turner, 1966; Distel *et al.*, 2011; Borges *et al.*, 2012). Shipworms (Mollusca: Bivalvia: Teredinidae; Fig. 2) are phylogenetically closely related to common mussels like *Mya arenaria* or *Macoma balthica*. However, body shape, life cycle, nutrition and habitat requirements are rather different in comparison to other marine bivalves.

At first glance, with the elongated body lacking a shell cover (Fig. 6), they are worm-like in their general appearance. This general appearance is the reason for the misleading name assignment 'shipworm'. The first nature observers like Aristotle and Pliny the elder perceived that these wood-destroying organisms are a kind of special marine species but they were not able to classify them. The meaning of the word 'shipworm' is ambiguous at this time and is used e.g. by Aristotle for wood-boring beetles and termites as well as shipworms (Moll, 1914). Therefore, it happened quite early that these organisms were sorted into wrong groups, usually to the insects (Moll, 1914). The first author who described them scientifically was Godofredus Sellius in his very detailed monograph *"Historia naturalis teredinis seu Xylophagi marini, tubulo-conchoidis speciatim belgici: cum tabulis ad vivum coloratis"* in 1733 (Sellius, 1733). He mentioned, however, that the famous personal doctor of the Queen of England Elisabeth I., Lister (*"Cochlearum Angliae Libri"*), was the first to place shipworms among mussels because of their intestines (Moll, 1914, 1928). Nowadays, there is no doubt about their taxonomic classification.

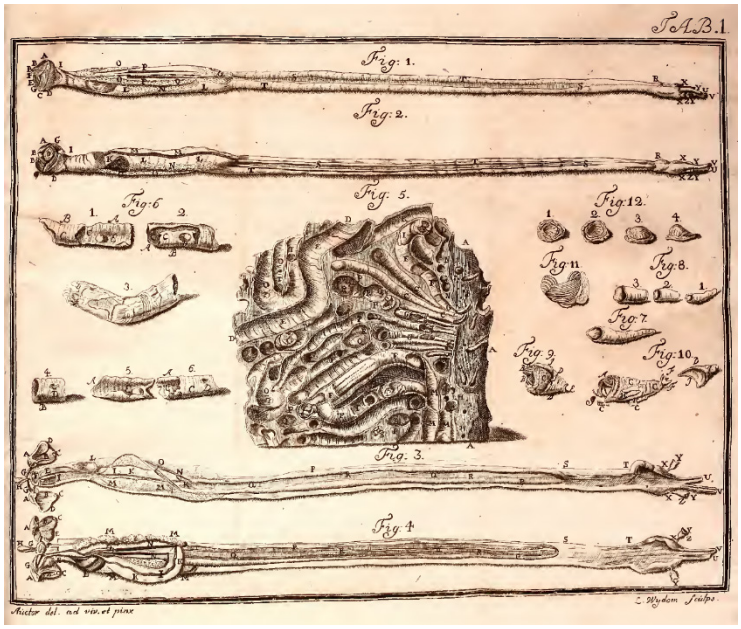


Figure 1 | Plate with the first known scientific illustration of shipworms sampled in the Netherlands, most likely *Teredo navalis*, made by Godofredus Sellius (Sellius, 1733).

For a long time, many authors (e.g. Roch, 1937) reported shipworm species numbers of up to 150 - 160 species. Moll (1942) even reported a list with 300 names of recent and 170 names of

fossil teredinids, but only containing half as many species. These numbers must certainly be considered with caution, as many scientific synonyms were given to the different species from various authors because of their great similarities and a lack of knowledge concerning their identification. For example, the WORMS database (World Register of Marine Species, http://www.marinespecies.org, 10.10.18) contains 16 different scientific synonyms for the common shipworm *Teredo navalis*, e.g. *Teredo beachi*, *Teredo japonica* or *Teredo sinensis*. It is confusing and not known whether these synonyms can really all be assigned to *T. navalis* or whether they might be distinct or even sibling species. Nevertheless, this phenomenon is also well-known for other shipworm species like e.g. *Lyrodus pedicellatus* (33 synonyms) or *Bankia carinata* (20 synonyms). It seems to be mainly caused by the difficult taxonomic determination of these wood-boring bivalves in contrast to organisms that do not drill into wood. Due to this possible overestimation, it can be questioned as to whether there are over 150 different species of shipworms. Today, the confirmed number is much lower. Various authors state the present known number of currently existing shipworm species of 68 (Calloway & Turner, 1988; Shipway, 2013). According to Voight (2015), there are 127 species of wood-boring bivalves (68 teredinids and 59 xylophagaids) in total that are known so far.

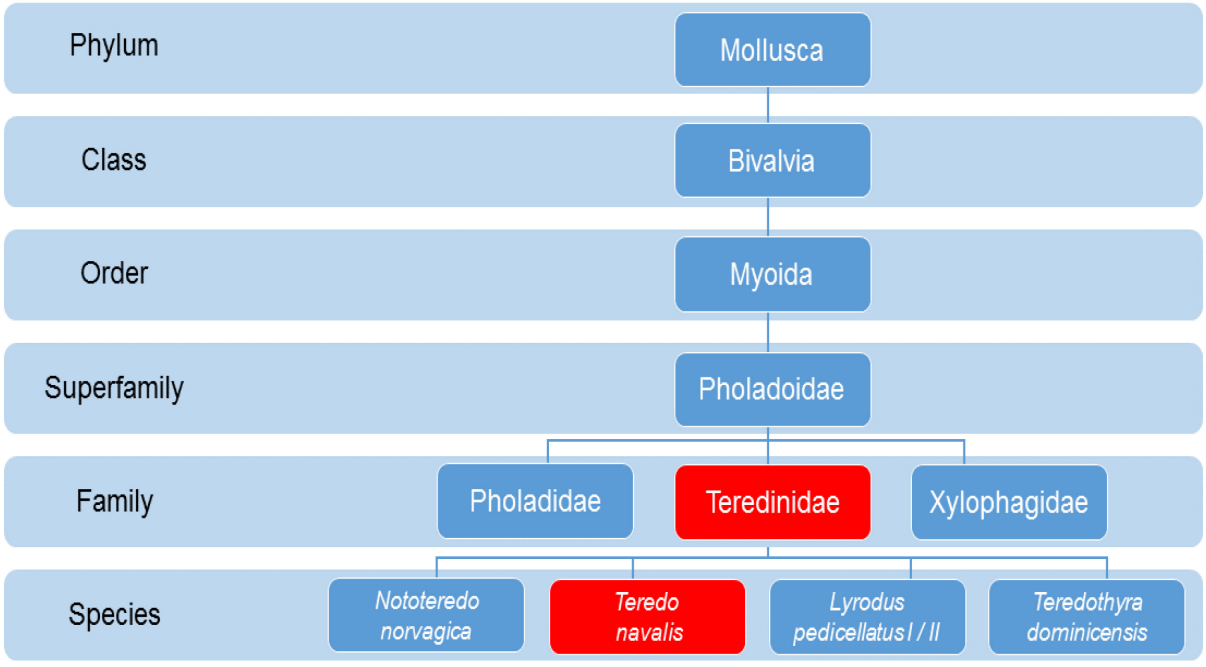


Figure 2 | Scheme for the taxonomical classification of *Teredo navalis*: The three other shipworm species (*Nototeredo norvagica*, *Lyrodus pedicellatus*, *Teredothyra dominicensis*) have been partly processed in the first publication of this thesis and are therefore mentioned here.

Biology

Overall, shipworms display the same basic anatomical structure as other mollusks such as clams and oysters. The predominantly biggest modification is the elongation of the body and the inner organs that represents an adaptation to the wood-boring way of living (Quayle,

1992). In adult shipworms, the location of the mouth and anus corresponds to the typical position of these organs in Bivalvia (Turner, 1966). The neural system is also typical for the Bivalvia and consists of three symmetrically arranged ganglia pairs: the merged pedals and visceral ganglia, as well as the cerebral ganglia (Moll, 1914).

Furthermore, there are some unique features that distinguish the teredinids from other Bivalvia, as described in detail by Turner (1966): "Other unique features in the anatomy of the Teredinidae include: 1) the anal canal, which extends from the anus over the visceral mass to the suprabranchial cavity; 2) the caecum, or wood-storing pouch, which extends posteriorly from the stomach (except in *Kuphus* which lacks a caecum); 3) the pallets with muscles which work them; and 4) the insertion of the siphonal retractor muscle on the calcareous lining of the burrow rather than on the valves from which they are, of course, greatly separated."

The structure of the shells resembles that of all Bivalvia and consists of three layers: Periostracum, Ostracum and Hypostracum (May, 1929). However, in contrast to all other Bivalvia, the shells do not enclose and cover the soft body of the animal. All Teredinidae are provided with a drilling unit that originated from the greatly reduced bivalve shell. This drilling unit starts to develop a few days after the settlement onto wood by the byssus filaments, once the metamorphosis from the free-swimming pediveliger larvae to the adult has taken place (Nair & Saraswathy, 1971). This way of life with planktonic larvae is found in all teredinids, albeit to varying degrees in the individual species.

The reproduction is diverse within the different species. Basically, there are three different methods of fertilization. Some species release the sex products via the excurrent siphon and the fertilization will take place in the water column. Other species show an internal fertilization process within the females with the subsequent development of the larvae in the breeding pouch (Nair & Saraswathy, 1971). Moreover, direct fertilization via the siphons of animals living next to each other has also been found. This so-called 'pseudocopulation' has been observed for the species *Bankia gouldi* (Clapp, 1951).

The adult phase is always benthic as animals are firmly attached to the living tube in the area of the entrance borehole (Hahn, 1956). As the entrance borehole is only 1 - 2 mm in diameter and the drilled living tube is always conical, the adults spend their whole life in this calcareous-lined tube (Nair & Saraswathy, 1971). However, the animals are able to contract in the tube to about one third of their body size (Grave, 1928; Roch, 1932). The maximum body length is limited by the inhabited wood but is indicated with up to 30 - 150 cm depending on the species (e.g. Grave, 1928; Nair & Saraswathy, 1971).

At the posterior end, two siphons for watering and draining as well as two hard calcareous structures, so-called pallets, and the connected muscles are located. The incurrent siphon is responsible for the supply of oxygen-rich water, plankton and the inflow of sperm cells, the

excurrent siphon for the removal of feces and the discharge of larvae (e.g. Roch, 1940; Hahn, 1956). The two siphons can be fully retracted and the borehole can be hermetically sealed through the pallets. Due to their shape, the pallets are well-suited to hermetically seal the living tube in case of danger or unfavorable environmental conditions. There are reports that this may take up to six weeks, depending on the species (Schütz, 1961).

The inner organs do not all find space between the two adductor muscles, which has led to a large prolongation of the gonads and gills. In special cells of the gills, the bacteriocytes, the xylotrophic teredinids contain symbiotic gram positive bacteria. Since the degradation product of the scraped wood chips mainly consists of cellulose and hemicellulose, which is difficult to digest, different endosymbiotic bacteria have been described for many teredinid species. These bacteria, living in symbiosis with the host, supply them with various enzymes such as cellulase and dinitrogenase. This enables the Teredinidae to use wood as their main food source and hence as a source of carbon (Distel *et al*., 2002a, 2002b).

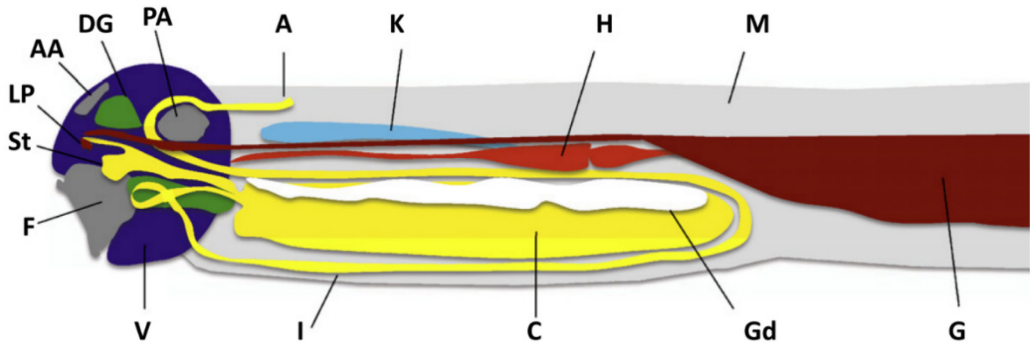


Figure 3 | General anatomy of a shipworm (Example: *Bankia setacea*): A) anus, AA) anterior adductor, C) caecum, DG) digestive gland, F) foot, G) gill, Gd) gonad, H) heart, I) intestine, K) kidney, LP) labial palps, M) mantle, PA) posterior adductor, St) stomach, V) valve, (Modified after Distel *et al*., 2011).

According to a study by Dore & Miller (1923), teredinids digest about 80 % of cellulose and 15 % to 56 % of hemicellulose, depending on the species of wood. This process does take place in the digestive tract whereas the bacteria are located in the gills. Therefore, the enzymes are required to be transported into the intestine in a hitherto unknown process (Distel *et al*., 2011).

Paalvast & van der Velde (2013) assumed that shipworms obtain their carbon mainly by filter feeding. However, the analysis of the carbon isotope ratios between five species of teredinids and the surrounding wood has shown that they selectively use cellulose, with wood being the most important carbon source of the Teredinidae (Nishimoto *et al*., 2009). That also invalidates the views of many former authors who have denied the nutritional value of wood and regarded drilling as a purely protective function (Dore & Miller and references therein, 1923). A common representative of these bacteria is the cellulolytic, dinitrogen-fixing bacterium

Teredinibacter turnerae. It was found in different wood-boring mollusks and seems to be the predominant symbiont of the Teredinidae (Distel, 2002a). An additional benefit of this symbiosis with bacteria is the air nitrogen-fixing capability of some of them. This makes the Teredinidae one of few animal groups that is able to compensate for a one-sided diet (Distel *et al*., 2011). In addition, as supplementary food, plankton can also be taken up from the surrounding water column via the incurrent siphon (Turner, 1961; Nair & Saraswathy, 1971). A pure plankton diet does not seem to be sufficient, as Roch's experiments with teredinids (*T. navalis*) outside the living tube have shown (Roch, 1932, 1940).

Habitats

The different species of Teredinidae are capable of tolerating a wide range of water temperatures and salinities. This is reflected in the above-mentioned widespread distribution from the cold Arctic waters through the temperate zone to the warm waters of the tropics. The preferred salinity usually ranges in the full-marine spectrum, although there are different species that populate other habitats, like brackish water (e.g. *T. navalis*) or even fresh water (e.g. *Nausitora dunlopei*).

Except for some completely different habitats inhabited by marine boring mussels, such as the rhizome of seaweed by *Zachsia zenkewitschi* or the benthos by *Kuphus polythalamia*, almost all members of the Teredinidae are mandatory wood-borers. They certainly require wood for building their living tubes, and almost all of them are obligate xylotrophic wood feeders (Turner, 1966). This ability to use wood as a source of food is unusual in the animal kingdom and is mostly known from the terrestrial environment. In the marine milieu, this part is mainly taken over by the wood-boring bivalvia (Distel *et al.*, 2011). In one of the first molecular biological studies on shipworms, the latter authors discovered through phylogenetic investigations of the nuclear loci 18S and 28S, that xylotrophy evolved only once in the class Bivalvia. However, for shipworms it is irrelevant whether the source of wood is of natural origin, such as mangrove roots or trees washed into the sea, or man-made such as ships or bridges. Due to its structure, elasticity and good price-performance ratio, wood is still widely used in the construction of harbors (piers, pile moorings, landing stage) and, above all, in coastal protection. In particular, the so-called groynes that are used to protect the shore from waves and currents are widespread, especially at the German Baltic Sea coast, where about 1023 rows with several tens of thousands of wooden groyne piles exist (M. Bugenhagen, StALU MM; pers. com.). Consistency seems to play a decisive role in the settlement of wood. Thus, it appears that the hardness of wood, the content of toxic alkaloids and silica are the most important factors (Bavendamm & Roch, 1970), with cellulose content and density also being relevant (Eriksen *et al*., 2017).

The drilled tubes are always lined with calcareous material, with the exception of the anterior end where further excavation takes place. The calcareous material is secreted from the surface of the body or mantle and is mainly used to protect the soft body of the animals (Grave, 1928). It can be replaced if the tube is damaged and the anterior burrowing can be sealed in that way too. This is an irreversible reaction to unfavorable environmental conditions such as timber limitation due to overpopulation (Grave, 1928). Even though an extremely large number of animals colonize a piece of wood it is not likely that another tube will be drilled (Nelson, 1922). The animals recognize existing tubes in proximity or the approximation to the end of the wood through a hitherto unknown sensory achievement and continue drilling in another direction (sometimes up to 180° in the other direction, own observation). However, the animals do not seem to use gravity as a mode of orientation as wood positioned vertically in the water is being drilled in both directions, upwards and downwards (Grave, 1928). This wood-boring way of life also includes a form of natural dispersal. Drilled into driftwood, almost all species are able to cover long distances with the prevailing water currents (e.g. Turner, 1966; Nair & Saraswathy, 1971; Scheltema, 1971). In this respect, a highly specialized species is *Uperotus clava*, which drifts long distances across the sea drilled only into the seed cases of *Xylocarpus granatum* mangroves (Roch, 1955; Voight, 2015).

Wood that has been attacked and destroyed by wood-borers represents a biocoenosis which has been poorly investigated so far. Roch (1931) reports several groups of animals (including e.g. polychaetes, bryozoans and crustaceans) associated with pieces of wood infested by *T. navalis* but did not distinguish between outer settlements and secondary use of abandoned *Teredo* tubes. Only few recent works dealing with this topic are known. In 2013 and 2014, Hendy *et al*. investigated the function of teredinids as ecosystem engineers in mangroves. It was found that the tunneled wood was used as refuge by different groups of animals (e.g. spiders, polychaetes, juvenile octopuses, small fishes) and that the temperature inside the infested wood was significantly lower than the outside air temperature. Teredinids, as ecosystem engineers, are therefore not only involved in the faster decomposition of wood but also actively influence their habitat by creating niches and habitats for nursery. Do they not only create an increased habitat complexity (Hendy *et al*., 2013) but also release nutrients by decomposing more than 50 % of the fallen wood (Voight, 2015). In regions inhabited by mangroves, the wood-boring mollusks are of great importance as ecosystem engineers in the decay of wood as they decompose wood faster than marine fungi or bacteria (Borges, 2007).

Historical background and previous distribution

From a historical and geological retrospect, the infestation of wood by teredinids is not a recent phenomenon as the Teredinidae are an evolutionary ancient animal group. The first precursors of the trees are already known from the Carboniferous period. With the development of seed plants, the trees widely spread in the Triassic (252.2 - 201.3 Mya) and reached a large number of species. This roughly corresponds with the emergence of shipworms that date back a few million years later. According to Moll (1942), the origin of the genus *Teredo* dates back to the Jurassic (~ 150 - 200 Mya) where they spread across the earth. Various authors report that fossilized residues of different teredinids or fossilized living tubes, so-called Teredolites, can be found in rock formations of the Jurassic period (Moll, 1942). Tauber (1954) describes *Teredo pulchella* as the oldest known species of teredinids from the Middle Jurassic in Europe, although no pallets were found. For the family Teredinidae, Distel *et al.* (2011) determined a monophyletic origin, which means that all recent species originated from only one common ancestor.

However, in the geological literature, almost all wood-boring species are only identified up to the genus level. For a specific identification, not only the fossilized living tubes but also the calcareous shells and pallets are needed (detailed description in chapter 1.2). Though, these are only to be found in some rare cases. Thus, a clear identification of fossils down to the species level is almost impossible. However, Tauber (1954) reports that numerous fossilized pallets were found in the area of the Vienna basin and the Eisenstädter Basin in Europe. For the first time, these findings have enabled a clear systematic classification of fossil teredinids and eight species or subspecies have been identified. Teredolites can also be detected in the sediments of the North Sea and the Baltic Sea (Moll, 1942; Schulz, 1995).

Sailors have been aware of the problem of wood-boring animals destroying harbor piers and ships for a long time. Especially the shipworms have always been suspected by seafarers and were feared as '*summa calamitas navium*' - a disaster for ships (Roch, 1935). Many descriptions have been found of how sailors have tried to protect their ships, for example by using copper plates or lead nails (Moll, 1914). Another method was to build double-walled ships and fill the interstitial space with e.g. charcoal or ash to prevent the inner shell from being attacked (Moll, 1914).

The first trustworthy records of shipworms date back to the authors of the classical antiquity of the area around the Mediterranean Sea (Roch, 1940). In this region, the Greeks and later also the Romans had already struggled with the infestation of their fleets by shipworms. There are also reports of infestations of parts of a Viking ship in the Haithabu Museum. Gollasch *et*

al. (2009) rule out retrospective infestation as the ship was found in fresh water. Nevertheless, it is not clear which species is responsible for the infestation.

Over the following centuries, the knowledge about shipworms seemingly decreased due to a lack of records (Moll, 1914; Hill & Kofoid, 1927). It was not until the 16th and 17th centuries that there was an increase in the amount of reports on shipworms in Europe. In particular, the frequent voyages of discovery to the various continents and the emerging trade relations brought along many reports of shipworms to Europe (Moll, 1914). From this time of the post-Columbian area, sailors told many stories about shipworms from their voyages to Europe and aroused interest in these organisms (Moll, 1914).

Since then, there have been various scientific publications covering the distribution, anatomy and physiology of shipworms in different parts of the world. This continues to date as numerous publications from the last decades demonstrate dealing with different aspects of these animals.

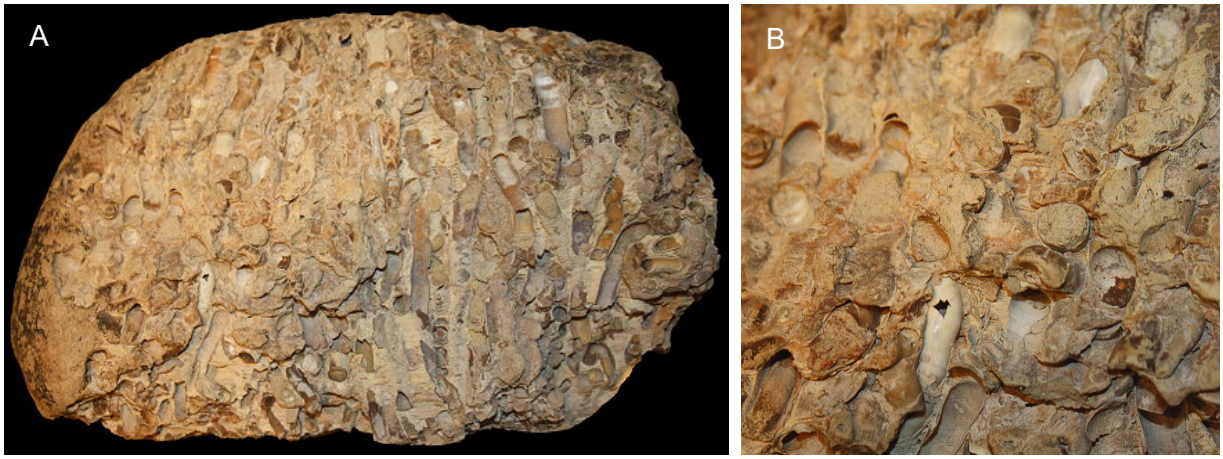


Figure 4 | A) Fossilized driftwood with tunnels of shipworms from the debris (Sternberg Rock). Location: Gravel pit Kobrow, Mecklenburg-Western Pomerania B). The calcareous linings of the burrows are still largely intact (enlarged details of picture A). © Paleontological Museum Nierstein

Recent distribution

Today xylotrophic shipworms are distributed worldwide, mainly in temperate and tropical regions (Borges *et al*., 2012). They occur in almost all seas and the number of presumptive species increases from the poles to the equator (Roch, 1955). Above all, the habitat range of shipworms from the family Teredinidae is restricted by salinity, temperature and the availability of wood as habitat and food source (Turner, 1966; Borges *et al*., 2012). For instance, there are four to five known shipworm species in Scandinavian waters and the North Sea, and about ten in the Mediterranean Sea. In the tropics, however, shipworms occur both in higher abundances and in larger species numbers. It is common for up to 20 species to occur in a single harbor (Roch, 1955).

Shipworms can also colonize Arctic waters, even though there are significantly fewer species with lower abundances. There are reports, both older and more recent, about occurrences (mostly *Psiloteredo megotara and Teredo norvagica*) in the areas around Greenland, Iceland and Svalbard (e.g. Roch, 1931). Many reports dealt with infested driftwood without knowing the original location of it, but there is evidence of wood which has been submerged at these sites and therefore must have been infested there (J. Berge, University of Tromsø; pers. com.). Another extreme habitat, the deep sea, is also inhabited by shipworms. Deep sea wood-borers belong to the family Pholadidae (subfamily Xylophagainae) which exclusively occur there (Turner, 2002; Distel *et al.*, 2011).

Figure 5 | Calcareous living tube of *Teredo navalis* with living animal inside.

There are some, though rare, reports of damages caused by shipworms in various regions. Contemporary estimates of up to 615 million US Dollar (in 1992 US Dollar) for damages to maritime structures in the Bay of San Francisco in the early years of the 20th century (Cohen & Carlton, 1995) and 50 million Euro for the Baltic Sea region (Wichmann, 2005) for the period from 1993 - 2005, to name a few. This is approximately in the order of magnitude caused by other marine invertebrate species such as *Dreissena polymorpha* by clogging of cooling pipes or by *Mnemiopsis leidyi*, which caused the entire fishing industry of the Black Sea to collapse in the 1990s.

1.2 *Teredo navalis*

Anatomy and morphology

There are few anatomical features specific to the genus *Teredo* some of which are briefly mentioned here. The gills are blade-like to u-shaped. The esophagus is short while the caecum is large and cylindrical. The elongated heart and gonads are located dorsal to the caecum. The feces are shaped as pellets and are transported through the posteriorly opened anal canal. The two siphons are separated from each other. By fusion of adjacent gill filaments, the brood pouch is formed at the dorsal surface of the gills (Turner, 1966).

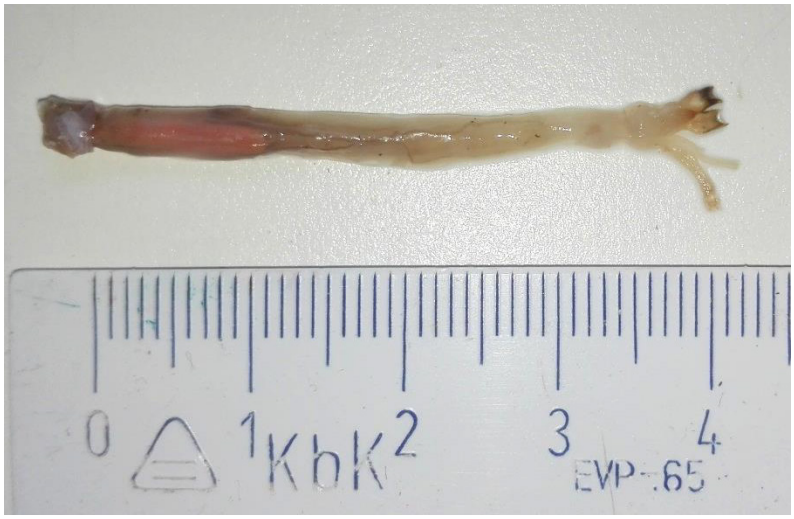


Figure 6 | Dissected specimen of *Teredo navalis* with drilling unit on the left side and pallets and siphons on the right side.

Reproduction and Life cycle

The species is a protandric hermaphrodite and can change its sex several times a year whereby the male characteristics are always developed first (Coe, 1941). Remarkably, the females always outnumber the males, even though the underlying process is not yet known (Moll, 1914; Grave, 1928). The gametes are released into the water column by the males and pass through the incurrent siphon into the adjacent female animals. The following fertilization is internal in the epibranchial chamber, where the comparatively small eggs (diameter ~ 50 - 60 µm) are brooded (Calloway & Turner, 1988).

Since the larvae are retained in this breeding pouch until they reach a length of approximately 100 µm, this type of breeding is called larviparous (Quayle, 1992). For the short brooding period in these chambers there are different data reported: they range from five (at 25 °C) to eight days (at 20 °C) (Calloway & Turner, 1988) up to a period of two to three weeks (Grave, 1928). Therefore, the species is called short-term larviparous (Borges *et al*., 2014b) and the larvae will be released at the straight-hinged veliger stage into the plankton (Shipway, 2013). Depending on the body size of the individual (Grave, 1928) the females release up to five million larvae per year (Kaestner, 1982). The development to the pediveliger stage continues in water for another two to four weeks depending on the water temperature (Calloway & Turner, 1988). Accordingly, Roch (1940) reported a free-swimming larval stadium of approximately two to three weeks. Similarly, Grave (1928) stated about five weeks for the complete larval period.

The largest infestation of wood with larvae seems to be about 50 - 100 cm above the mud line (Grave, 1928). When the larvae are attached to a piece of wood by the byssus filaments at an approximately length of 250 µm (Quayle, 1992), the velum gets lost and a metamorphosis follows. The bivalve shells hitherto enclosing the body are converted into a drilling unit and

the larva starts drilling. The growth takes place rapidly and two to three weeks after settling the organisms already reach a size between 0.35 and 1 mm. Within 30 days, lengths of up to 7 mm and a diameter of 2.5 mm are reached. Growth continues to reach a length of up to 75 mm and up to 4.5 mm in diameter after 60 days (Grave, 1928). The size of the adults in the North Sea and Baltic Sea is stated 10 to 20 cm (Hahn, 1956) or rather 20 to 25 cm with a maximum of up to 30 cm (Schütz, 1961). The information for the life span in this region varies between one to two and a half (Schütz, 1961), one to three (Nakel, 1954) and finally two to three years (Hahn, 1956). After a relatively short generation time of 40 - 45 days after metamorphosis (Grave, 1942) and a length of approximately 3 - 5 cm they become sexually mature. Sordyl *et al*. (1998) reported developed eggs already in specimens of 2 cm body length and larvae from 3 cm upwards. There is no known periodicity in spawning of this species presumably taking place all year round (Grave, 1928). Therefore, three to four generations per year are possible under favorable conditions (Coe, 1941). The temperature range for spawning activities is differently specified by various authors. Grave (1928) reported spawning for the US East Coast region when water temperatures between 11 and 12 °C are reached. Several other authors, however, state that there is no reproduction below 15 °C. There are various spawning temperatures given in the literature such as 15 °C or 18 °C, respectively temperature ranges of 15 - 16 °C or 15 - 20 °C (Nair & Saraswathy and references therein, 1971).

Ecophysiology of *Teredo navalis*

Salinity

Several authors specify an optimum salinity range from 7 to 35 for species development (e.g. Blum, 1922; Nair & Saraswathy, 1971). The reported maximum salinity that adults are able to tolerate ranges between 35 (Nair & Saraswathy, 1971) and 39 (Borges *et al*., 2014b). However, adults always tolerate lower salinities than the larvae (Nair & Saraswathy, 1971). The adults show a normal behavior with regard to the drilling activity and mobility of the siphons down to a salinity of 9 (Blum, 1922). The drilling activity of *T. navalis* is completely stopped below a salinity of 7 and the required minimum salinity for the survival of adults is 5 - 6. Other authors reported a lethal salinity of 4 - 5, respectively (Nair & Saraswathy and references therein, 1971). If the salinity drops further below the limit of 5 - 6 during longer periods the siphons are completely retracted and the tubes are sealed with the pallets (Blum, 1922). *Teredo navalis* can survive these low salinity conditions for up to six weeks without major effects on vitality as can be detected when salinity returns to the optimal range (Roch, 1932; Schütz, 1961). A salinity of around 10 was reported to be harmful to the larvae (M´Gonigle, 1926 in Nair & Saraswathy, 1971). In the Baltic Sea, the species tolerates salinities down to 8 and is,

surprisingly and for the first time observed, still able to reproduce in this brackish water conditions (Sordyl *et al.*, 1998).

Temperature

Various studies state that the optimum temperature for the development of *T. navalis* is between 15 - 25 °C while the tolerated temperature range is between 5 - 30 °C (e.g. Roch, 1932; Nair & Saraswathy, 1971). Outside this range, survival is still possible but with limitations. Even a short-term increase of temperature to above 30 °C leads to a rapid mortality of all individuals (Roch, 1932). Laboratory experiments have shown that the larvae drilled most actively when the temperature was between 17 - 22 °C and did not drill below 14 °C or above 26 °C (Imai in Norman, 1976b).

Once the temperature decreases below 9 to 10 °C the metabolism is reduced and the activity of the siphons is severely restricted. Below 5 °C the siphons are completely retracted, drilling activity is stopped and the tube is being sealed with the pallets (Roch, 1932). Temporary decreases in temperature down to 0 °C or even -1.4 °C cause inactivity that, however, can lead to a recovery in case of a change to appropriate conditions (Nair & Saraswathy, 1971). Only in cold icy winters with long periods below 0 °C it might happen that the organisms are extinguished (Roch, 1932).

Interspecific interactions

Due to their wood-boring way of life, shipworms are hardly threatened by natural enemies. Various groups of organisms, such as annelids, protozoa and bacteria are named (e.g. Nair & Saraswathy, 1971) as potential enemies of *T. navalis*. However, due to good protection provided by the almost hermetically sealed living tubes these seem to be only scavengers. However, it cannot be ruled out that attacks on shipworms occasionally occur.

As one of a few exceptions, Grave (1928) mentioned the protist *Architophrya* (a holotrich) which is said to attack *T. navalis* to death when the housing tube is damaged. Nakel (1954) reports of the worm *Nereis fucata* that was found only in the empty tubes of *T. navalis*. This behavior was also observed on another polychaete (*Alitta succinea*) in the Baltic Sea (own observation).

Taxonomic classification

The reason why *T. navalis*, according to various authors, is the most important species of shipworms can be explained by its scientific name. The genus name *Teredo* originates from the Greek word '*teredon*' and means 'wood worm'. The species name *navalis* means 'living in ships'. Hence, the species name *Teredo navalis* means 'the wood worm living in ships'.

Consequently, this has been the most common synonym for shipworm species having been detected in ships for a long time. This is well reflected in a wide variety of non-scientific names *T. navalis* has to date: 'shipworm', 'common shipworm', 'naval shipworm', 'great shipworm', 'European pileworm' (all English), '*paalworm*', '*gewone paalworm*' (Dutch), '*almindelig pæleorm*' (Danish) '*Pfahlwurm*', '*Pfahlmuschel*', '*Bohrwurm*' and '*Schiffsbohrwurm*' (German), just to name a few.

Linnaeus performed the first taxonomic classification in 1758. The specimens for this classification were collected in the Netherlands by Sellius in the early years of the 18th century. But Linnaeus made a mistake. In the 10th issue of the '*Systema naturæ*' he categorized *Teredo* into the so-called '*Intestina*' (roundworms) and did not correct this error until the 12th issue. Even then did he class them with the single-walled shell animals as he thought the calcareous tube was a main part of the animal and identified *Teredo* as a kind of tube snail (Roch, 1931). Aggravating for Linnaeus was that he only knew one species while Spengler mentioned four different species in 1792 and Jeffreys described 18 species in 1869 (Roch, 1931). Although there may be more synonyms for other shipworms, *Teredo navalis* is presumably the best known. If a single species name like *T. navalis* was synonymously used for a whole group of slightly different species (Moll, 1914) it is challenging to trust records or animal descriptions from former times.

Species identification of shipworms is difficult. Although there are several characteristics such as the grain of the siphons or the number of the small teeth on the shell, the most widely used is the shape of the calcareous pallets (Turner, 1966; Borges *et al*., 2012). These pallets (Fig. 7, 8 A, 8 B) consist of a stalk, which sticks in a membranous handle sheath, and a pallet blade (Roch, 1940). Depending on the species, the stalk is oval, round or flat. Both the length and the shape (e.g. curved, straight, thickened) are different. The blade, too, can be very diverse in form. In the genus *Teredo* they usually look like paddles or spoons with a forked end. In addition, the pallets, which are usually convex on the outside and concave on the in-

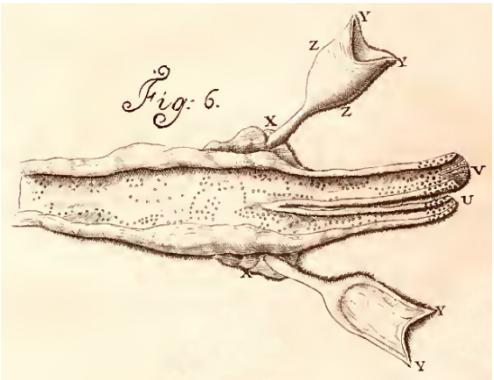


Figure 7 | First detailed illustration of the *Teredo navalis* pallets by Godofredus Sellius (Sellius, 1733).

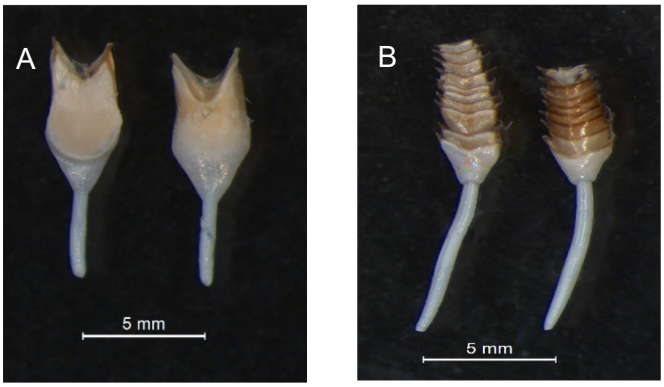


Figure 8 | A) Pallets of *Teredo navalis* from the sampling site Kiel. B) Pallets of the Genus *Bankia* from a sample from the Mediterranean Sea.

side, are often fitted with two small channels into which the two siphons can be retracted (Roch, 1940). The first more or less complete - and still the best - systematic species identification key was written by Turner (1966). Although even with this reference book, a reliable identification is often not possible, since it is very difficult to prepare the filigree pallets as a whole. However, only the large groups such as e.g. *Bankia* (segmented) and *Teredo* (unsegmented) can be easily distinguished from each other as the pallets have different shapes (Fig. 8 A, 8 B). The shape of the pallets can also differ between juveniles and adults within a species or between animals of different regions. For example, subspecies such as *Teredo navalis borealis* in Scandinavian waters have also been described but were later retracted (Roch, 1931).

Apart from the difficulties described above, these misidentification are partly due to the fact that species identifications have been exclusively based on the analysis of morphological characteristics so far. To be prepared to allocate damages and spreading to a certain species it is necessary to have a fast, accurate and reproducible way of shipworm species identification. Recently, some attempts have been made to use modern molecular taxonomic approaches. This so-called DNA barcoding approach (Hebert *et al.*, 2003) has successfully been used for the identification of shipworm species (*Teredothyra dominicensis*; Shipway *et al.*, 2014) and the identification of a putative species complex (*L. pedicellatus*; Borges *et al.*, 2012). Borges *et al.* (2012) tried to combine the morphological taxonomy with the molecular taxonomical DNA barcoding approach. This approach has worked for some shipworm species (e.g. *L. pedicellatus*, *N. norvagica*), but not for *T. navalis* (L. Borges, pers. com.). For this reason, *T. navalis* was not accessible at this time with regard to this molecular species identification method.

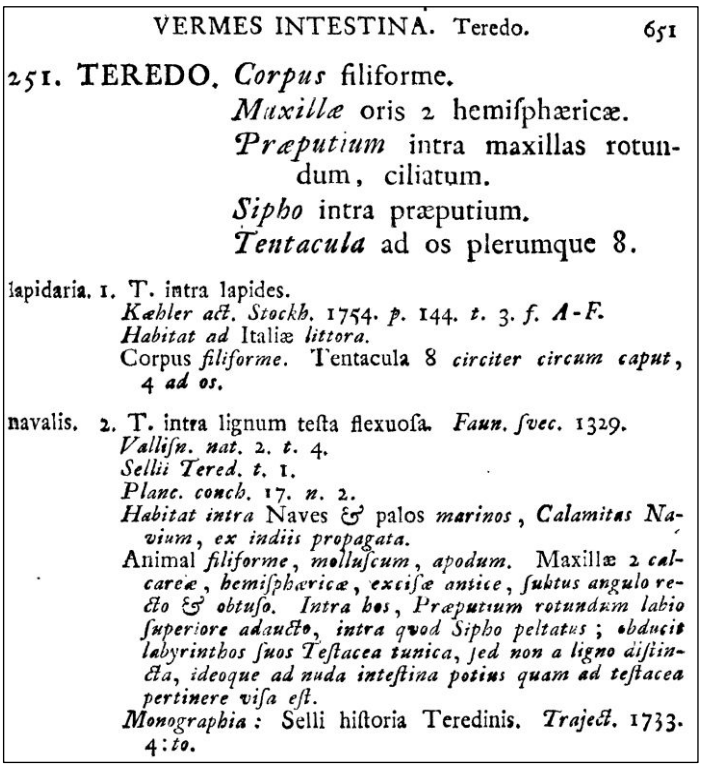
Therefore, one of the major aims of this thesis was to develop specific molecular tools for an unambiguous identification of *T. navalis*.

Historical and known recent distribution in Europe

Since the classical antiquity, the word ‘τερηδών’, which means ‘the boring one’, has been known for all wood-boring pests (Roch, 1940). The Latinized form ‘*Teredo*’ was then received by the Romans through Ovid and Pliny and was retained in later years by Sellius and Linnaeus for the scientific naming (Roch, 1931, 1940). From this antique period, in one of these writings Ovid (*Ex Ponto, Liber primus*, verses 69 - 74 in Roch, 1935) compares 'the immensity of his soul anguish with the ship-destroying activity of *Teredo*' (Roch, 1940). However, the first reliable mentions of the species *T. navalis* for the Mediterranean Sea were not made until 1792 by the Italian author Giuseppe Olivi (Roch, 1940). In 1829, the author Delle Chiaje described three different species (presumably *T. navalis*, *T. utriculus* and *Bankia*

carinata) from the area around Naples. Prior to that, probably all shipworms in the Mediterranean were referred to as *T. navalis* (Roch, 1940).

Moll (1940) described *T. navalis* as the cosmopolitan species of the cold temperate zone, while *L. pedicellatus* is supposed to be the equivalent for the warm temperate zone. Nowadays, several authors regard *T. navalis* as a species that is distributed worldwide (e.g. Turner, 1966; Appelqvist *et al.*, 2015a). Based on Turner's data, Nair & Saraswathy (1971) identified six contiguous regions for the occurrences of *T. navalis*: Australia/New Zealand, Southeast Asia, Japan, West Coast of North America, East of North America/Greenland and Europe/Atlantic Coast. There are various references for this worldwide distribution: Australia (Ibrahim, 1981), Japan and India (Tsunoda, 1979; Ibrahim, 1981; Kasyanov *et al.*, 1998), Persian Gulf and South Africa (Moll, 1940), Brazil (Barreto *et al.*, 2000), Mexico (Naranjo-Garcia & Castillo-Rodriguez, 2017), the West (Ibrahim, 1981; Cohen & Carlton, 1995) and East Coasts (Ibrahim, 1981, Culliney, 1975) of North America, the Atlantic, Baltic Sea, North Sea, Black Sea and Mediterranean Sea in Europe (see e.g. Tuente *et al.*, 2002; Culha, 2010; Borges *et al.*, 2014b). In terms of its origin, however, *T. navalis* still has to be considered cryptogenic, since the place of its origin is still unknown (Hoppe, 2002).



VERMES INTESTINA. Teredo. 651

251. TEREDO. *Corpus* filiforme.
Maxillæ oris 2 hemiſphæricæ.
Præputium intra maxillas rotundum, ciliatum.
Sipho intra præputium.
Tentacula ad os plerumque 8.

lapidaria. 1. T. intra lapides.
Kæhler act. Stockh. 1754. *p.* 144. *t.* 3. *f. A-F.*
Habitat ad Italiæ *littora.*
Corpus *filiforme.* Tentacula 8 *circiter circum caput*, 4 *ad os.*

navalis. 2. T. intra lignum teſta flexuoſa. *Faun. ſvec.* 1329.
Valliſn. nat. 2. *t.* 4.
Sellii Tered. t. 1.
Planc. conch. 17. *n.* 2.
Habitat intra Naves & palos *marinos*, *Calamitas Navium*, *ex indiis propagata.*
Animal *filiforme*, *molluſcum*, *apodum.* Maxillæ 2 *calcareæ*, *hemiſphæricæ*, *exciſæ antice*, *ſubtus angulo recto & obtuſo. Intra hos*, *Præputium rotundum labio ſuperiore adaucto*, *intra quod Sipho peltatus*; *obducit labyrinthos ſuos Teſtacea tunica*, *ſed non a ligno diſtincta*, *ideoque ad nuda inteſtina potius quam ad teſtacea pertinere viſa eſt.*
Monographia: Selli hiſtoria Teredinis. *Traject.* 1733. 4:*to.*

Figure 9 | Facsimile of the original species description of Linnaeus (1758).

The first trustworthy sightings of *T. navalis* for Central European waters are reported for the Netherlands. They date back to the early years of the 18th century and coincide with the great flood in the Netherlands (1731) which was caused by shipworm attacks of the wooden parts of the dykes. These infestations were assigned to *T. navalis* from several authors (e.g. Vrolik *et al*., 1860; Van Benthem Jutting, 1943). Further sightings from this region date back to the years 1770, 1827 and 1858/59. For the further eastern area of Cuxhaven the first report on shipworm infestation dates back to 1791 (Woltmann 1791 in Kühl, 1972). There was also a report of the collapse of a wooden dam in January 1860 in Wilhelmshaven, which is attributed to *T. navalis* and has considerably delayed the construction of the naval base (Gollasch *et al.*, 2009).

The first scientific record of *T. navalis* for the Baltic Sea dates back to the year 1835 (Meyer & Möbius, 1865). Specimens of this species as well as *Nototeredo norvagica* were noticed in the region of the river Schlei in the northern Bay of Kiel. Afterwards, an infestation of the sluice area of the Eider Canal (predecessor of Kiel Canal) was discovered in 1872 (Möbius 1872 in Schütz, 1961) and a large distribution was also reported in the Kiel Fjord. In this area, a lot of wood from the coastal protection structures was infested (Schütz, 1961).

According to Kühlmann (1909, in Sordyl *et al*., 1998), the first report for the coast of Mecklenburg-Western Pomerania dates back to 1875 for the region around Rostock. At that time, coastal protection structures were infested which were built around 1860 in the area of Rostock/Warnemünde. However, the infestation with shipworms does not seem to have been of great extent at that time. In a manual of dune reconstruction from this time only other wood-boring species (crustaceans) than *T. navalis* were described (Cordshagen, 1964). Another record of the infestation of wooden structures with shipworms at the coastline of Mecklenburg-Western Pomerania dates back to 1917 and was reported by the port builder of the Rostock harbor Kiecker (Cordshagen, 1964). Noteworthy occurrences of shipworms in this area were also recorded for the years 1925 - 1928 with the infestation of the eastern bridges of Warnemünde (Nakel, 1954). Some years later, several authors reported a spread of *T. navalis* in the hot summers of 1933 - 1936 up to the area of the Darss Sill (Becker, 1938; Nakel, 1954). In the subsequent period up to 1993, only two minor infestations occurred in 1951 and 1976 (Schulz, 1995).

For the following years up to 1993, there have been no reports of significant damages or occurrences of shipworms at the coast of Mecklenburg-Western Pomerania (Sordyl *et al*., 1998). Nevertheless, the situation in the southern Baltic Sea has changed fundamentally since 1993. Since then, occurrences of a wood-boring bivalve species in this area have been documented and last until today. Whereas in the past (before 1993) only sporadic occurrences of shipworms were reported, there has been evidence of a stable, self-reproducing population since 1993 (Sordyl *et al*., 1998). This new situation was of crucial importance since far-reaching consequences with regard to coastal protection maintenance depend on this occurrence of shipworms. Therefore, it is important for stakeholders and decision makers of coastal protection measures to be able to identify the species involved quickly and reliably and to know present distribution boundaries. In this context, the cooperation with the State Agency for Agriculture and Environment of Central Mecklenburg (StALU MM), which is responsible for the coastal protection management in Mecklenburg-Western Pomerania, and the idea of this thesis have arisen.

Figure 10 | Different levels of the destruction of groynes. A) An undamaged groyne field. © StALU B) A groyne field with lots of broken groyne piles. © StALU C) A broken groyne pile, weakened by heavy infestation of *Teredo navalis*. D) Cross-section of an infested groyne pile with visible living tubes of *Teredo navalis*.

1.3 Aims

In the early years of the 20th century, a lot of research has been done on shipworms in general and on *T. navalis* in particular. There are, however, many open questions, regarding taxonomical identification of wood-boring bivalves, places of origin of different species and genetic population structures of shipworms. Studies using a modern molecular biological approach are rare and still missing on *T. navalis*.

The present thesis focuses on European waters and the Baltic Sea in particular. With the establishment of a permanent and self-reproducing shipworm population in the Baltic Sea, a new situation has risen in 1993. Compared to previous occurrences in recent decades it is still unclear whether only one species is responsible for the ongoing destruction of wooden constructions or if several species or even sibling species inhabited the world's largest brackish sea. Furthermore, the potential of the established shipworm species to expand further into waters of lower salinities is under discussion.

Therefore, three main aims should be clarified within the present thesis:

1. Is the dominant shipworm species in the Baltic Sea indeed *T. navalis*, as commonly accepted, or do other shipworm species or sibling species of *T. navalis* occur?

2. Is the Baltic Sea population of the identified wood-boring bivalve species genetically structured and clearly differentiated from populations outside the Baltic?
3. Where is the present distribution boundary of the occurring shipworm species located in the Baltic Sea and is there a range expansion into areas with lower salinities during the last decades?

Well-founded knowledge of these issues is a key factor in understanding the shipworm species composition in the Baltic Sea, its present distribution and the potential range expansion of shipworms in the future under changing environmental conditions. This knowledge can ultimately lead to sustainable coastal protection management and a better protection of valuable underwater heritage. Therefore, the present thesis aims to combine a modern molecular taxonomic with an established ecological approach to address the issues that are of great importance to decision makers.

2 Materials and methods

Study area

Seven locations at the coast of Mecklenburg-Western Pomerania were selected for the ecological investigations of *Teredo navalis* along a decreasing salinity gradient from West to East in the Baltic Sea (Boltenhagen, Kühlungsborn, Graal-Müritz, Zingst, Hiddensee, Dranske, Glowe; Fig. 1 in chapter 3.3). In agreement with the StALU MM, this sampling took place directly in the groyne fields of the southern Baltic Sea. From these locations, a subset of five sampling sites (Kühlungsborn, Graal-Müritz, Zingst, Hiddensee, Dranske) was chosen for genetic analyses. Further samples for phylogenetic and population genetic analyses originate from other parts of the Baltic Sea, from the Belt Sea, the North Sea, the Mediterranean Sea and the Atlantic Ocean (for details see Figure 11).

Sampling strategy

Specimens were sampled using test panels consisting of local pine (*Pinus sylvestris*, 10 x 20 x 2.5 cm) exhibiting a size according to DIN EN 275 (DIN, 1992). This method is commonly used and has been proven suitable for sampling teredinids by several authors (e.g. Borges *et al*., 2014b, Eriksen *et al*., 2014). Test panels were exposed at all sampling sites introduced above. Directly after their removal out of the water the test panels were photographed and cleaned of fouling before further processing.

Figure 11 | Map with an overview of all sampling sites. MS, Mediterranean Sea; US, North American East Coast BR, Brittany; ED, Emden; WA, Wangerooge; SY, Sylt; TJ, Tjärnö; KR, Kristineberg; LY, Lynaes; KI, Kiel; BH, Boltenhagen; KB, Kühlungsborn; GM, Graal-Müritz; ZI, Zingst; HI, Hiddensee; DR, Dranske; GW, Glowe.

Ecological investigation

From 2012 to 2015, test panels were placed at the seven stations introduced above and collected after defined periods according to the research question. The sampling stations were chosen along the prevailing salinity gradient in the Baltic Sea between Boltenhagen in the West with the highest and Glowe in the East with the lowest salinity. At all sampling sites a set of test panels was deployed at the beginning of the field campaign in April/May and was collected in October/November to determine the total larval abundances. In addition, test panels were changed monthly at four selected sampling sites (Boltenhagen, Kühlungsborn, Zingst, Dranske) to determine the time period of larval settlement. At the end of the field campaign, test panels were once again applied at all stations to prove possible latecomers during late autumn/winter.

At the four sampling sites of Boltenhagen, Kühlungsborn, Zingst and Dranske (Fig.16) temperature and salinity were recorded simultaneously in the groyne fields to directly determine these two key factors. The autonomous salt water HOBO Conductivity Logger (HOBO - U24-002, Onset Ltd., Cape Cod, Massachusetts, USA) independently recorded temperature and salinity hourly all year round. Data were downloaded every four weeks while cleaning the devices from fouling. Both the test panels and the measuring instruments were fixed with cable ties directly to the groynes at approximately 0.5 m above sea floor level. For details *cf.* material and methods in chapter 3.3. Subsequently, detectable boreholes were counted in the laboratory using a stereomicroscope.

Handling of specimens

For the classical and the molecular taxonomic identification, both juvenile and adult animals were taken of each sampling site. Until further processing, these samples were transferred to 96 % ethanol or stored at -60 °C. A complete list of all specimens examined for this thesis is given in the supplementary material of the respective publications including corresponding sampling sites and GPS coordinates.

PCR and Sanger sequencing

The samples for these analyses were collected during two periods between 1999 - 2000 and 2011 - 2016. For information, that is more detailed *cf.* the material and methods sections of the respective publications in chapter 3.1, 3.2 and 3.3.

For a molecular taxonomic identification of the occurring shipworm species in the Baltic Sea, several COI primers were developed (*cf.* material and methods in chapter 3.1). For this purpose, the DNA barcoding approach propagated by Hebert *et al.* (2003) was used. This in-

volves the use of a certain section of the COI gene for the molecular taxonomic determination. These newly established primers were used for both PCR and Sanger sequencing. The number and designation of these primers as well as the respective primer sequences are shown in Table 1 of chapter 3.1. All sequences were determined in the own laboratory by Sanger sequencing. The sequences of the different genetic markers were checked manually, aligned and trimmed to an uniform length. All obtained sequences were deposited to NCBI GenBank. Detailed information about GenBank accession numbers, sequence IDs, corresponding datasets and a complete list of COI haplotype titles are given in the supplementary material of the respective publications.

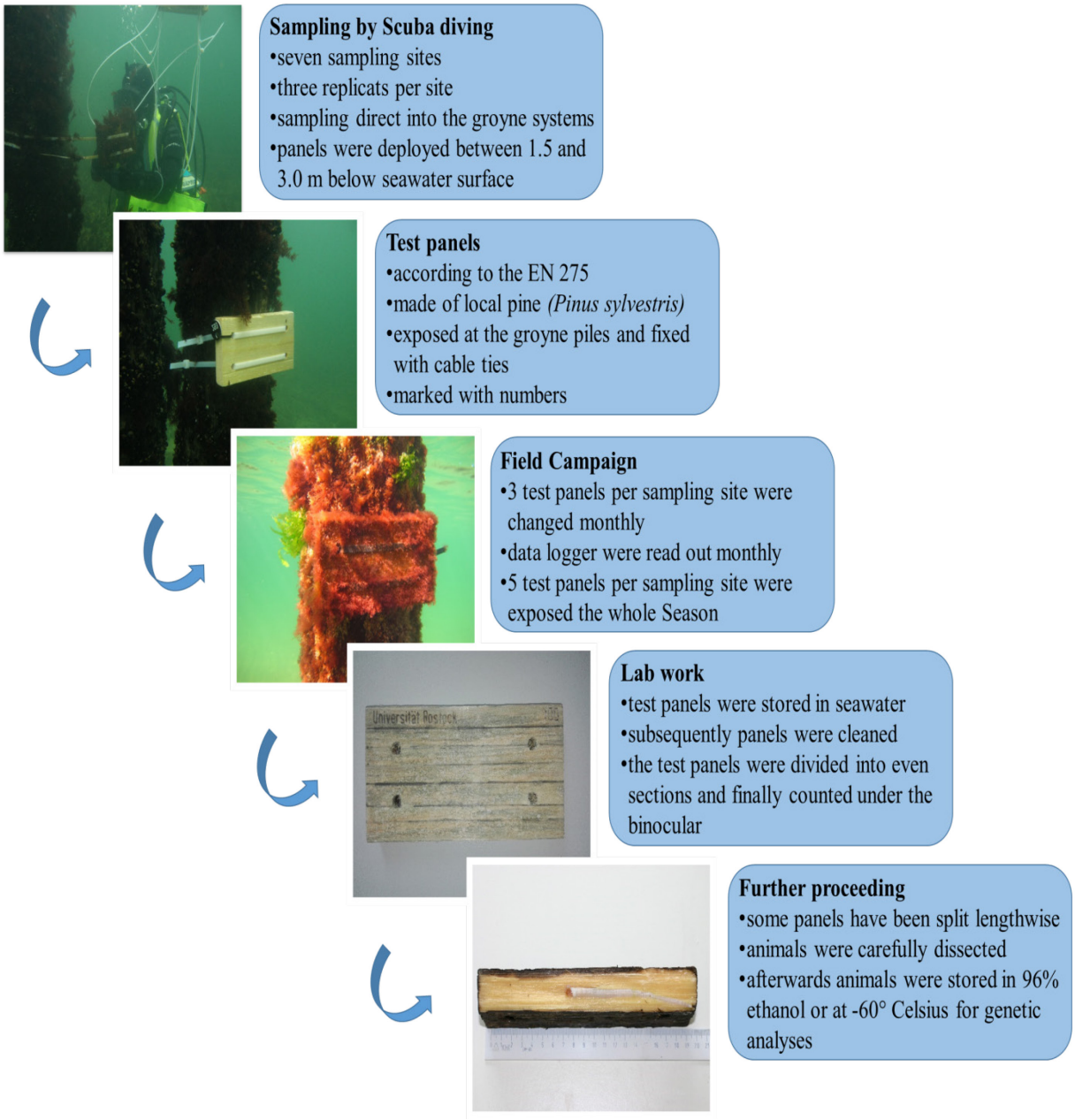


Figure 12 | Flowchart with the individual steps during the field campaign and in the laboratory.

Phylogenetic calculations and population genetic analyses

The determined alignments of the respective gene loci were used for different phylogenetic calculations. For this purpose, the alignments were converted into different file formats according to the various phylogenetic and population genetic calculation programs used. All used programs and calculation methods are described in detail in the material and methods sections of the respective publications of chapter 3.1 and 3.2. The results were mainly displayed graphically and are shown in the results sections of chapters 3.1 and 3.2.

3 Publications

3.1 First time DNA barcoding of the common shipworm *Teredo navalis* Linnaeus, 1758 (Mollusca: Bivalvia: Teredinidae): molecular-taxonomic investigation and identification of a widespread wood-borer

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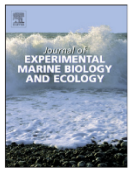

First time DNA barcoding of the common shipworm *Teredo navalis* Linnaeus 1758 (Mollusca: Bivalvia: Teredinidae): Molecular-taxonomic investigation and identification of a widespread wood-borer

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ABSTRACT

The common shipworm *Teredo navalis* is one of the most widespread marine wood-boring bivalves of the world and probably one of the most wood destructive and cost-incurring marine invertebrates. First reports on *T. navalis* for Europe date back to 1731 for the North Sea (The Netherlands) and to 1835 for the Baltic Sea (Germany). It is still unclear, however, where this species originates from. Therefore, *T. navalis* is considered cryptogenic for European waters, including the Baltic Sea.

In this study, 181 specimens of *Teredinidae* from six different sampling areas all over Europe and North America were molecular-taxonomically investigated using several molecular markers, two nuclear (18S/28S) as well as one mitochondrial marker (cytochrome c oxidase subunit I, hereafter COI). For the COI gene amplification, a new specific primer pair (Ter fw II/Ter rev I) for *T. navalis* was developed, which allowed sequencing of a 675 bp COI gene fragment for the first time. For amplifying the COI gene fragment of other examined teredinids than *T. navalis*, a third primer (Ter fw III) was designed. These three new primers are valuable tools to identify teredinid species with the DNA barcoding approach.

Classification of *T. navalis* into the system of wood-boring bivalves using a combined 18S/28S dataset showed no differentiation between specimens from Europe and the North American East Coast. The results of the COI dataset analyses showed high haplotype diversity in combination with a low nucleotide diversity and a star-shaped network with a predominant haplotype occurring in all investigated regions. Moreover, no indications have been found on a sibling species in the Baltic Sea. The data indicate a recent population expansion for the examined sampling sites whereas the origin of the assumed worldwide distributed species *T. navalis* remains open.

1. Introduction

Marine wood-boring Teredinidae (commonly known as shipworms or teredinids) occur in almost all aquatic ecosystems from tropical to cold-temperate waters (Turner, 1966). At least nine wood-boring bivalves have been reported so far in European coastal waters (Borges et al., 2014) of which four have been examined in the present study. Most of these species occur predominantly in fully marine conditions instead of brackish waters (Borges et al., 2012). The monophyletic group of wood-boring molluscs (Distel et al., 2011) inhabits all types of wood including mangrove roots and drift wood but also anthropogenic structures like docks or groynes which are used in coastal protection (Roch, 1937; Nakel, 1954; Hahn, 1956). These xylotrophic organisms perform an important ecosystem function by decomposing wood faster than the decay caused by marine fungi and bacteria (Borges, 2007). On the other hand, these bivalves cause enormous economic costs by damaging wooden coastal protection structures and harbour infrastructure. This is leading to substantial maintenance costs worldwide on an annual scale (Distel et al., 2011). The German Federal Maritime and Hydrographic Agency, for example, estimated economic damages up to 25 million Euro between 1993 and 2004 (BSH, 2004) for the Baltic Sea. In addition, these bivalves are also threatening invaluable underwater cultural heritage because the Baltic Sea hosts up to 100,000 well-preserved shipwrecks and other maritime related constructions of high archaeological value (Björdal et al., 2012).

Based on morphological treatments, the dominant wood-borer in the Baltic Sea was identified as the worldwide distributed common shipworm (also referred to naval or great shipworm) *Teredo navalis*

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0022-0981/

(Mollusca: Bivalvia: Teredinidae) Linnaeus 1758 (Sordyl et al., 1998; Appelqvist et al., 2014). According to several authors, *T. navalis* reaches a maximum length up to 30 cm and a lifespan up to three years in the Baltic Sea (Nakel, 1954; Hahn, 1956; Schütz, 1961). This species is a protandric hermaphrodite species with rapid sexual maturity 7–8 weeks after metamorphosis (Grave, 1942). A single female adult can release up to five million larvae per year into the water column (Kaestner, 1982), and therefore has a very high reproductive potential. The planktonic veliger larvae remain in the water column up to 34 days before settlement (Nair and Saraswathy, 1971). In the Baltic Sea, *T. navalis* tolerates salinities as low as 7 (Blum, 1922; Nair and Saraswathy, 1971; Hoagland, 1986; Strömberg and Spicer, 2003, Borges et al., 2014). The optimum temperature for *T. navalis* is between 15 and 25 °C. At temperatures less than 10 °C metabolic activities decelerate and stop at temperatures below 5 °C (Roch, 1932). Furthermore, the average winter water temperatures in the Baltic Sea do not have lethal effects for adults (Sordyl et al., 1998).

Based on the data of Turner (1966), Nair and Saraswathy (1971) have specified the worldwide distribution of *T. navalis* for six regions: Australia/New Zealand, Southeast Asia, Japan, West Coast of North America, East of North America/Greenland and Europe/Atlantic Coast. Trustworthy proofs supporting this distribution are rare. Nevertheless, there are references of the occurrence of *T. navalis* for Australia (Ibrahim, 1981), Japan and India in Asia (Tsunoda, 1979; Ibrahim, 1981; Kasyanov et al., 1998), Brazil in South America (Barreto et al., 2000), the West (Ibrahim, 1981; Cohen and Carlton, 1995) and East Coasts (Ibrahim, 1981, Culliney, 1975) of North America and the Atlantic, Baltic Sea, North Sea, Black Sea and Mediterranean Sea in Europe (see e.g. Tuente et al., 2002; Culha, 2010; Appelqvist et al., 2014; Borges et al., 2014).

While for most European waters like the Mediterranean Sea or the North Sea *T. navalis* has been reported long before 1800 (Streftaris et al., 2005), the earliest known report of this species for the Baltic Sea dates back to 1835 in the Bay of Kiel (Meyer and Möbius, 1865). Afterwards, *T. navalis* had been reported on the southern coast of the Baltic Sea in 1875 and in the 1920s (Sordyl et al., 1998). Subsequently several periodic mass outbreaks with increased abundances have been documented in the Baltic Sea (Germany, southern Sweden and Denmark) during the 1930s and 1950s lasting each for 2–3 years only (Bavendamm and Schmidt, 1948; Nakel, 1954; Hahn, 1956; Schütz, 1961). These periodic outbreaks between the early 20th century and the 1990s seem to be a special characteristic in the Baltic Sea, probably due to the strong salinity gradient from West to East in the Baltic Sea (Leppäranta and Myrberg, 2009).

Nevertheless, it is unclear whether *T. navalis* occurred between these outbreaks in the Baltic Sea, for example, as population with low specimen numbers or as stable, but simply undetected population in those years. Between these mass outbreaks, *T. navalis* is not mentioned in the literature. One of the problems related to 'invisible' wood-boring species is, that they will only be registered if occurring in great masses or causing great damages. In addition, there is also a lack of scientific investigations or regular monitoring programmes dealing with *T. navalis* at those years in the Baltic Sea. Therefore, reliable data about the abundances of *T. navalis* are missing for the Baltic Sea.

But this has greatly changed with the last mass outbreak in 1993 and the observed reproducing adult animals at the coast of Mecklenburg–Western Pomerania (Sordyl et al., 1998). The latest outbreak is well documented because of the establishment of a more or less regular monitoring of groynes over the last 20 years by the local State Agency for Agriculture and Environment of Central Mecklenburg (StALU MM). In contrast to earlier times, it seems that *T. navalis* established a stable, reproductive population present in the brackish south-western part of the Baltic Sea (Sordyl et al., 1998; Lippert, unpublished results). Sordyl et al. (1998) observed for the first time all stages of gonad development as well as free living larvae in the water column. In addition, for several years, there is evidence that *T. navalis* has been spreading into lower salinity brackish waters of various coastal regions in Europe which were formerly not inhabited by this species (Sordyl et al., 1998; Tuente et al., 2002; Paalvast and van der Velde, 2011; Borges et al., 2014). These observations might be related to global change phenomena such as e.g. increasing water temperatures (Neumann, 2010; Störmer, 2011).

The species name *T. navalis* is often used as a synonym for all kinds of wood-boring bivalves mainly due to lack of taxonomic knowledge about most other species of this group/family and the difficulties to identify them morphologically (Turner, 1966; Borges et al., 2012). Consequently, the place of origin and the bio(phylo)geography of *T. navalis* remain still unsolved. Therefore, this taxon is considered to be cryptogenic (Hoppe, 2002; Gollasch et al., 2009; Borges et al., 2014). Even less is known about the pathways of introduction of *T. navalis* into the Baltic Sea. To address any type of range expansion or bioinvasion, an unambiguous taxonomic identification of the species of interest is absolutely essential. Nevertheless, morphological identification of bivalve wood-boring species often fails. This may be partly related to their way of life as they live hidden in a calcified tube drilled into the wood. Therefore, there are associated difficulties to extract the animals unharmed in one piece out of the tubes. The only known morphological features for accurate taxonomic identification are exclusively represented by two calcareous pallets, located at the distal end of the elongated body (Turner, 1966; Borges et al., 2012), which, however, are easily lost or destroyed during preparation. Molecular taxonomy such as barcoding is thus an appropriate approach to solve these taxonomic difficulties within wood-boring bivalves and has already been successfully used for the identification of other teredinids (Borges et al., 2012).

Molecular taxonomic identification of animals by using the DNA barcoding approach started in 2003 (Hebert et al., 2003). Although there are some challenges with this DNA barcoding (see Collins and Cruickshank and references therein, 2013) it is now accepted as a reliable, standardized, cost-effective and widely-used method for biodiversity investigations on all life stages (Hebert et al., 2003; Radulovici et al., 2010). It includes sequences from the 5′ region of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene as marker for species-level identification. Although the BOLD system (barcode of life data system; www.boldsystems.org; Ratnasingham and Hebert, 2007) contains actually more than 4×10^6 (15.05.2015) data entries, appropriate data on *T. navalis* are missing. The closest related species with COI sequences within GenBank and the BOLD system are *Lyrodus pedicellatus* and *Bankia carinata*. In addition, until now there are only three sequences of *T. navalis* deposited in the NCBI GenBank (18S/28S and actin gene). No COI sequences are available yet (15.05.2015), probably because the universal COI primers of Folmer et al. (1994) fail to amplify this mitochondrial marker in this species (Borges, pers. com.).

Therefore, one of the aims of the present study was to develop specific COI primers for *T. navalis* as a new molecular tool for reliable taxonomic identification of this species. Afterwards, the hypothesis was tested whether occurring Teredinidae in the Baltic Sea are exclusively represented by *T. navalis*, as commonly accepted, or by better adapted sibling species with respect to now well established and self-reproducing populations in contrast to previous years. The occurrence of sibling species has ecological and evolutionary implications and they are common in all major marine groups and habitats (Knowlton, 1993). The phenomenon of sibling species which occurs in the same regions has been documented for related teredinid species (Calloway and Turner, 1983; Borges et al., 2012) and in other marine invertebrates (Bastrop and Blank, 2006).

Furthermore, also the hypothesis that *T. navalis* is a species with an amphi-Atlantic distribution was verified. Hence, this study tried to clarify the phylogenetic relationship of the European *T. navalis* in the general phylogeny of wood-boring molluscs by using two nuclear markers (18S and 28S) as used in the study of Distel et al. (2011). To outline whether there are differences between other teredinid species from Europe and from North America, available samples from three of

the eight other teredinid taxa described for Europe (*L. pedicellatus*, *Nototeredo norvagica*, *Teredothyra dominicensis*) were additionally examined phylogenetically.

2. Material and methods

2.1. Sampling and storage

A total number of 181 wood-boring specimens of the family Teredinidae were collected over a prolonged period between 1999 and 2012 in six different sampling areas [Europe (5), East Coast of North America (1)] (Fig. 1). Most sampling areas comprise several sampling sites. The majority of specimens collected originate from the area of the North Sea and the Baltic Sea. A complete list of individual samples used in this study as well as an overview of all sampling sites and GPS coordinates is given in Table S1 in the supplementary data of the online version.

Specimens were collected from permanent sources like groyne piles as well as from deployed test panels. All wooden structures used for sampling consisted of non-durable pine wood (*Pinus sylvestris*) which is suitable for sampling teredinids (DIN EN 275, 1992; Borges et al., 2014). Each specimen represents one individual belonging to the teredinid subfamilies Teredininae or Bankiinae. Taxonomic nomenclature is according to Turner (1966, 1971). Samples from 1999 and 2000 were stored frozen at −60 °C and later transferred into 96% ethanol. All other samples were directly stored in 96% ethanol after preparation prior to further DNA analysis.

In addition, for phylogenetic tree calculation, 32 18S rDNA as well as 32 28S rDNA sequences from different bivalve species were used from NCBI GenBank from the study of Distel et al. (2011). Therefore, in the present study specimens of all three Teredinid subfamilies were represented (Teredininae, Bankiinae, Kuphinae) as well as representatives of the families Myidae, Corbulidae and Dreissenidae as reference taxa used as outgroups for phylogenetic tree calculations. For detailed information see Table S2 in the supplementary data.

2.2. DNA extraction, amplification and sequencing

Total DNA was extracted from mantle and muscle tissue using an 'innuPREP Forensic Kit' (Analytik Jena) following protocol five (user manual). The final elution volume was 200 µl. Large and small subunit nuclear rRNA genes (18S and 28S) as well as the mitochondrial COI fragment were amplified using polymerase chain reaction (PCR) performed with different amplification parameters. All PCR reactions were performed with an Analytik Jena FlexCycler. Primer pairs as well as amplification parameters used for 18S/28S and COI PCR are listed in Table 1.

For phylogenetic tree calculation and for pairwise distance calculation, the 18S gene fragment was amplified for several specimens of all four teredinid species (*T. navalis* (3), *L. pedicellatus* (4), *N. norvagica* (3) and *T. dominicensis* (2); number of specimens in brackets), the 28S gene fragment, however, was amplified for only one specimen of each species. The nuclear rRNA genes consist of an approximately 1700 bp section for small (18S) subunit and a 1300 bp section for large (28S) subunit. PCR was performed in a 30 µl reaction volume consisting of 10× PCR buffer, 2.0 mM $MgCl_2$, 1.5 U Moltaq (Molzym GmbH & Co. KG), 62.5 µM dNTP each, 1 pmol of each 18S/28S primer and 3 µl DNA template.

Tests with universal primers LCO1490 and HCO2198 (Folmer et al., 1994) as well as several other bivalve primers did not work for *T. navalis*. Nevertheless, for few specimens of *T. navalis* a 900 bp gene fragment was successfully amplified and sequenced by using the Mat fw and Mat rev primer pair (Matsumoto, 2003) (Table 1). From these fragments the new COI primers Ter fw II and Ter rev I (Table 1) were developed, which worked well for all analysed 172 specimens of *T. navalis*. For amplifying the nine other teredinid specimens, a third primer (Ter fw III; see Table 1) was designed. Together with Mat rev, this primer pair worked well for the three other examined species *L. pedicellatus*, *N. norvagica*, and *T. dominicensis*. For phylogenetic network calculation and pairwise distance calculation a 675 bp fragment of the COI gene of all 181 teredinid specimens was amplified. PCR was performed in a 30 µl reaction volume consisting of 10× PCR buffer, 4.0 mM $MgCl_2$,

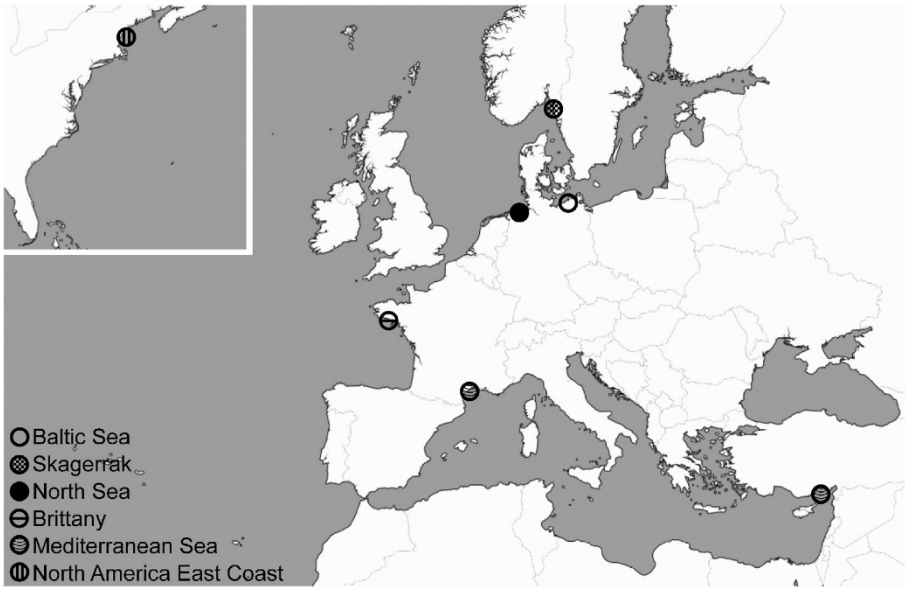

Fig. 1. Sampling areas in Europe and North America (detailed in the upper left corner). More explicit information about the sampling areas and sampling sites can be found in the supplementary data of the online version.

Table 1
Primers used for PCR as well as for sequencing and PCR amplification parameters for amplification of nuclear and mitochondrial genes (same superscript numbers denote PCR primer pairs).

Gen	Primer name	Primer sequence (5′ → 3′)	Sense	References
18S	18S-fw[1]	CCTAYCTGGTTGATCCTGCCAGT	Forward	Englisch and Koenemann (2001)
	18S-rev[1]	TAATGATCCTTCCGCAGGTT	Reverse	Englisch and Koenemann (2001)
	18F509	CCCCGTAATTGGAATGAGTACA	Forward	Struck et al. (2002)
	18L	GAATTACCGCGGCTGCTGGCACC	Reverse	Struck et al. (2006)
Amplification parameter	Initial denaturation 94 °C, 5 min; 35 cycles: 94 °C for 30 s, 52.5 °C for 30 s, 72 °C for 200 s; final extension 70 °C, 5 min			
28S	28S-NFL 184-21[2]	ACCCGCTGAAYTTAAGCATAT	Forward	www.psb.ugent.be/rRNA
	28S-1600R[2]	AGCGCCATCCATTTTCAGG	Reverse	Distel et al. (2011)
	28S-D5CF	ACACGGACCAAGGAGTCT	Forward	Park and O'Foighil (2000)
	28S-D4RB	TGTTAGACTCCTTGGTCCGTGT	Reverse	Park and O'Foighil (2000)
Amplification parameter	Initial denaturation 95 °C, 2 min; 35 cycles: 95 °C for 60 s, 55 °C for 60 s, 70 °C for 60 s; final extension 70 °C, 5 min			
COI	LCO 1490[3]	GGTCAACAAATCATAAAGATATTGG	Forward	Folmer et al. (1994)
	HCO 2198[3]	TAAACTTCAGGGTGACCAAAAAATCA	Reverse	Folmer et al. (1994)
	Ter-fw II[4]	GTTTCTGGTTAATGCCTAGATC	Forward	This study
	Ter-rev I[4]	GCTCTGGATACGACAATCCCAG	Reverse	This study
	Ter-fw III[6]	GG(A/G/T)GATAT(A/G)GC(T/C)TT(T/C)CCTCG	Forward	This study
	Mat fw[5]	AT(T/C)GG(A/T/C/G)GG(A/T/C/G)TT(T/C)GG(A/T/C/G)AA(T/C)TG	Forward	Matsumoto (2003)
	Mat rev[5, 6]	AT(A/T/C/G)GC(A/G)AA(A/T/C/G)AC(A/T/C/G)GC(A/T/C/G)CC(T/C)AT	Reverse	Matsumoto (2003)
Amplification parameter	Initial denaturation 94 °C, 2 min; 37 cycles: 94 °C for 20 s, 52 °C for 20 s, 72 °C for 60 s; final extension 72 °C, 5 min			

0.75 U Moltaq (Molzym GmbH & Co. KG), 62.5 μM dNTP each, 1 pmol of each COI primer and 3 μl DNA template.

Thereafter 5 μl of each PCR product was visualized in 1% agarose gel under UV light. Clear PCR products were purified with an 'innuPREP Gel Extraction Kit' (Analytik Jena) and used for sequencing after a second control electrophoresis.

Sequencing of PCR products was performed using the dideoxy chain termination method and cycle sequencing (Sanger et al., 1977) using an ABI Prism® Big Dye® Terminator V.1.1 Cycle Sequencing Kit'. Primers used for sequencing are listed in Table 1. Cycle sequencing products were analysed by using capillary separation on an ABI Genetic Analyzer 3130 xl, Applied Biosystems/Hitachi. All products were sequenced in both directions and all sequences obtained in this study are deposited in GenBank. For detailed information of all used sequences, including the GenBank accession numbers, see Table S1 in the supplementary data of the online version.

2.3. Phylogenetic analyses

Recorded data were manually checked and aligned with Bioedit (Hall, 1999) using the ClustalW algorithm. Phylogenetic analyses were performed using MEGA 6 (Tamura et al., 2013) and Network (Bandelt et al., 1999). MEGA 6 was used for maximum likelihood (ML) and for pairwise distance calculation. Pairwise distance analyses were conducted using the Kimura 2-parameter model (K2P). Haplotype diversity and nucleotide diversity were calculated with DnaSP (Librado and Rozas, 2009).

For calculating the phylogenetic network using the median-joining method of Network, an alignment of 675 bp with all 172 determined COI sequences of *T. navalis* was generated. A complete list of all haplotypes with sampling sites and GenBank accession numbers is shown in Table S1 in the supplementary data of the online version.

The combined 18S/28S dataset consists of 2817 bp (incl. gaps). For phylogenetic tree calculations, an alignment with a total of 36 combined sequences was created and finally aligned with MUSCLE (www.ebi.ac.uk). The sequences of four European wood-boring bivalves *T. navalis*, *L. pedicellatus*, *N. norvagica* and *T. dominicensis* investigated in the present study, were (with authorization) included into the alignment of Distel et al. (2011). The partition homogeneity test (Cunningham, 1997) was done by Distel et al. (2011) with 100 replicates to evaluate the validity of combining datasets. For ML calculations, the best fit model of evolution was determined by the implemented modeltest in MEGA 6 (TN93 + G + I). The GenBank accession numbers of all examined genes are shown in Tables S1 and S2 in the supplementary data.

3. Results

3.1. Combined 18S/28S dataset

All four examined European teredinid species fit very well into the phylogeny of wood-borers proposed by the previous study of Distel et al. (2011) and belong to the Teredinidae, which can be divided into the two subfamilies Teredininae (*T. navalis*, *L. pedicellatus*, *T. dominicensis*) and Bankiinae (*N. norvagica*). In the present study, *N. norvagica*, which has been missing in the phylogeny of Distel et al. (2011), forms together with *Dicyathifer mannii* a sister group of *Teredora malleolus*. European *T. dominicensis* clustered with a sample of the same species from South America. Together they are a sister group of *Kuphus polythalamia*, which formed a sister group with *T. malleolus* in the previous phylogenetic tree.

All examined 18S as well as 28S sequences of *T. navalis* from Europe match exactly with the one from the North American East Coast (Distel et al., 2011). As shown in the phylogenetic tree, *T. navalis* represents a sister group of the *L. pedicellatus* specimens from California (*L. pedicellatus* CA), the Atlantic (*L. pedicellatus* I) and the Mediterranean Sea (*L. pedicellatus* II). The Atlantic *L. pedicellatus* I is more closely related to that specimen from California than to the one from the Mediterranean Sea. The results show also that the sequences of *L. pedicellatus* FL from Florida form a cluster with sequences of *Lyrodus massa*. Both species together are a sister group to all other *Lyrodus* species as well as to *T. navalis*.

Pairwise distances of 18S sequences showed no intraspecific divergences (for specimens collected in the same area) (Table 2). The species *L. pedicellatus* I from the Atlantic, however, diverged 0.06% from *L. pedicellatus* II collected in the Mediterranean Sea. The

Table 2
Pairwise distances in % (K2P) of nuclear 18S gene within and between all investigated species. Averaging over all sequence pairs are shown. The analysis involved 12 nucleotide sequences and a total of 1710 positions in the final dataset. All positions containing gaps and missing data were eliminated (n: number of specimen, n.a.: not applicable).

		Pairwise distances (18S)				
		Within	Between species			
	Species name (n)		1	2	3	4
1	*Teredo navalis* (3)	0.00				
2	*Lyrodus pedicellatus* I (3)	0.00	0.06			
3	*Lyrodus pedicellatus* II (1)	n.a.	0.12	0.06		
4	*Nototeredo norvagica* (3)	0.00	1.96	2.02	1.96	
5	*Teredothyra dominicensis* (2)	0.00	2.14	2.20	2.20	2.26

species showing the greatest divergences from all other species with ~2% are *N. norvagica* and *T. dominicensis*.

3.2. COI dataset

The COI gene fragments of *T. navalis* specimens were obtained by PCR for the first time in the present study. The alignment consists of a total number of 675 bp without any gaps or missing data. The total number of mutations is 73 with 71 polymorphic sites. A total of 64 haplotypes were determined. The 64 identified haplotypes are referred to as haplotype Tn1 to Tn64 in the network (Fig. 3). The distribution of the different haplotypes from each sampling site is shown in Table S1 in the supplementary data. The calculated haplotype diversities ranged between 0.714 (Mediterranean Sea) and 1.000 (North American East Coast) (Table 3). At this point, it should be noted that the sample size for North America was very low (n = 2) which may have caused this high value. The nucleotide diversity looks somewhat different with the lowest value in the Mediterranean Sea (0.00169) and the highest value in the North Sea (0.00253).

Both, the intraspecific as well as the interspecific pairwise distances of the COI sequences were considerably larger than the corresponding 18S distances from the same specimens. The maximum intraspecific differences for the COI gene diverged from a minimum of 0.19% (*T. navalis*) and a maximum of 0.39% (*N. norvagica*). The results of interspecific pairwise distances are similar to those from the 18S distances. The COI sequences of *L. pedicellatus* I from the Atlantic diverged from those collected in the Mediterranean Sea (18.80%). The greatest divergences from all other species were again found for *N. norvagica* (32.64%) and *T. dominicensis* (31.88%) (Table 4).

For deeper investigation of phylogenetic relationships among haplotypes of *T. navalis*, a median-joining network analysis was done. The most common and ancestral haplotype (Tn1) was found in each of all six sampling areas and consists of 82 individuals (Fig. 3). Consequently, this haplotype was carried by 47.7% of all individuals sampled. The topology of the network showed this haplotype to be located in the centre of a star-shaped network encircled by numerous rare haplotypes. Most of these haplotypes occurred at very low frequencies and in total there were 48 private haplotypes (Fig. 3). Only two of these private haplotypes consisted of more than one specimen (Tn11, Tn45).

The second most common haplotypes were Tn9 (Baltic Sea, North Sea, Brittany) and Tn57 (North Sea, Skagerrak) each representing 6 individuals (~3.5%). The third most frequent haplotypes represented also three (Tn55; Skagerrak, Brittany, Mediterranean Sea) and two (Tn10; Baltic Sea, North Sea) areas respectively, with three individuals each (1.7%). In total 18 haplotypes were found in two or more specimens. Consequently, 46 haplotypes consisted only of sequences of one single specimen distributed over all sampling areas (except North American East Coast). Most haplotypes (57.8%) differed from the dominant haplotype only by one mutation step. The longest distance between two different haplotypes was eight mutation steps.

Table 4
Pairwise distances in % (K2P) of mitochondrial COI gene within and between all investigated species. Averaging over all sequence pairs are shown. The analysis involved 12 nucleotide sequences and a total of 675 positions in the final dataset (n: number of specimen, n.a.: not applicable).

		Pairwise distances (COI)				
		Within	Between species			
	Species name (n)		1	2	3	4
1	*Teredo navalis* (3)	0.19				
2	*Lyrodus pedicellatus* I (3)	0.00	21.93			
3	*Lyrodus pedicellatus* II (1)	n.a.	21.57	18.80		
4	*Nototeredo norvagica* (3)	0.39	32.64	28.91	32.21	
5	*Teredothyra dominicensis* (2)	0.00	30.57	30.16	30.83	31.88

4. Discussion

The taxonomic classification of the European wood-boring molluscs *T. navalis*, *L. pedicellatus*, *N. norvagica* and *T. dominicensis* into the phylogenetic system of bivalves, as postulated by Distel et al. (2011), supports the phylogenetic hypothesis of monophyly of the Teredinidae (Fig. 2). Thus, the gathered data of the present study confirmed the proposed phylogeny of the previous study with some minor variations.

For *L. pedicellatus*, a divergence of ~0.6% in the 18S pairwise distances between specimens from the Atlantic and the Mediterranean Sea was observed. This is confirmed by the COI pairwise distance analysis (18.8% divergence) and similar to the value obtained by Borges et al. (2012). For this reason, the latter authors suggested that *L. pedicellatus* from the Atlantic and the Mediterranean represent two putative species. The present study followed this suggestion, and assigned specimens of *L. pedicellatus* from the Atlantic as *L. pedicellatus* I and specimens from the Mediterranean Sea as *L. pedicellatus* II. The classification of the *L. pedicellatus* specimen from Florida is different to that from Distel et al. (2011) and confirms the results obtained by Borges et al. (2012). The latter authors postulated that *L. pedicellatus* from Florida might be *Lyrodus floridanus*, which is supported by the data that have been presented in the Results section. Turner and Calloway (1987), however, displayed the difficulties in the taxonomic classification of *L. pedicellatus* and *L. floridanus* because of similar pallets, but different life cycles. A final determination can only be provided by a combined approach of classic taxonomy and molecular biology. It is also remarkable at this point that the genus *Bankia* seems to be non-monophyletic (Fig. 2). The same could be seen in the phylogenetic analyses of Distel et al. (2011), and also Borges et al. (2012) mentioned that *Bankia* might be another case of putative cryptic species. The European specimens of *T. dominicensis* and *N. norvagica* fit well into the phylogenetic system of Distel et al. (2011) but will not be discussed further.

As shown in the Results section, the 18S and the 28S sequences from Europe match exactly with those from the North American East Coast examined both in the present as well as in the previous study from Distel et al. (2011). The species *T. navalis*, as far as is known, occurs in most European coastal waters and is together with *N. norvagica* the

Table 3
Haplotype diversity of mitochondrial COI gene of *Teredo navalis* within different sampling areas and in total. The analysis involved 172 nucleotide sequences and a total of 675 positions in the final dataset. n: number of sequences used, S: polymorphic (segregating) sites, H: number of haplotypes (private haplotypes in brackets); Hd: haplotype diversity (with variance and standard deviation), π: nucleotide diversity (with variance and standard deviation).

	Haplotype analyses of different *Teredo navalis* sampling areas								
	n	S	H	Hd	Variance (Hd)	SD (Hd)	π	Variance (π)	SD (π)
Baltic Sea	55	29	23 (14)	0.723	0.00463	0.068	0.00192	0.0000001	0.00034
North Sea	65	45	32 (22)	0.813	0.00253	0.050	0.00253	0.0000001	0.00032
Skagerrak	23	14	14 (5)	0.822	0.00691	0.083	0.00180	0.0000001	0.00032
Brittany	20	14	11 (5)	0.763	0.01064	0.103	0.00221	0.0000002	0.00049
Mediterranean Sea	7	4	4 (2)	0.714	0.03272	0.181	0.00169	0.0000004	0.00062
North American East Coast	2	1	2 (0)	1.000	0.25000	0.500	0.00148	0.0000005	0.00074
Total	172	71	64	0.771	0.00126	0.035	0.00215	0.0000000	0.00019

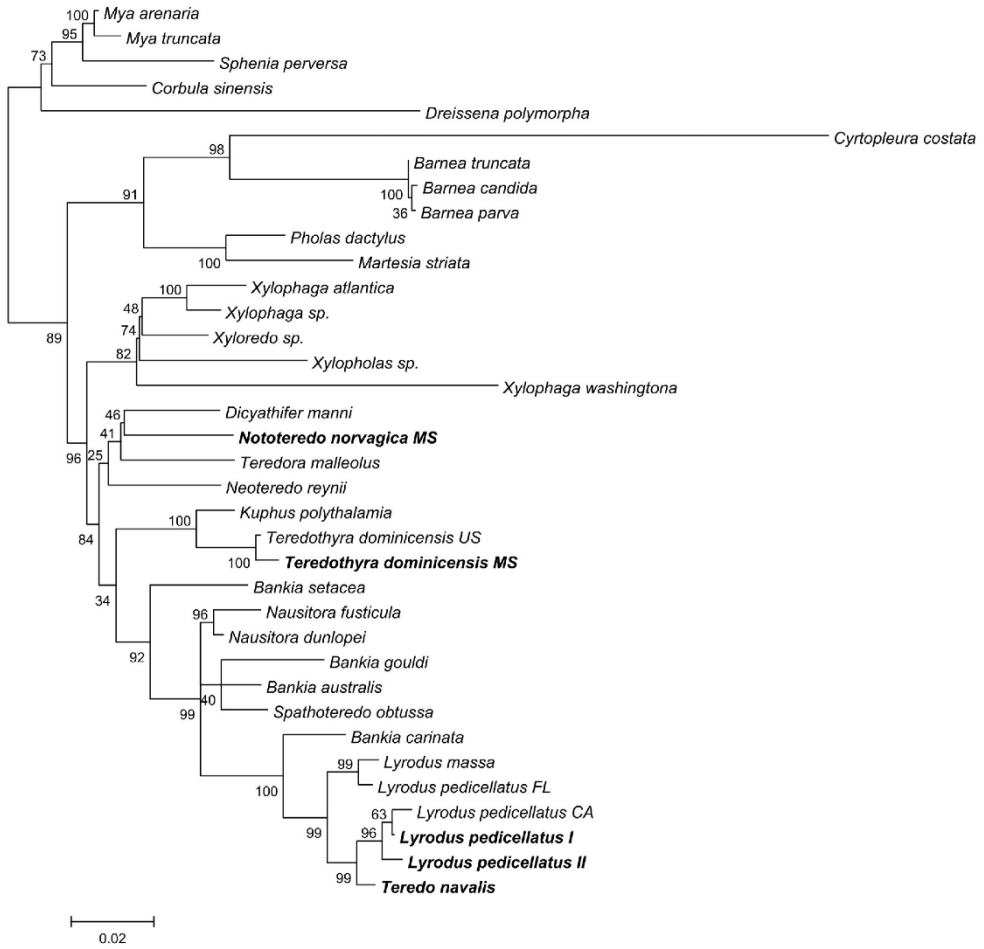

Fig. 2. Phylogenetic tree hypothesis of 18S/28S combined dataset of wood-boring bivalves and related taxa. 36 nucleotide sequences involved in the final dataset. Maximum likelihood (ML) calculation based on the Tamura–Nei model (TN93 + G + I) of a 2817 bp (incl. gaps) alignment. Abbreviations: CA — California, FL — Florida, MS — Mediterranean Sea, US — North American East Coast. Bold species names denote sequences determined in this study.

species with the widest distribution range in Europe (Borges et al., 2014). It is, for example, the most abundant teredinid species in Swedish waters (Appelqvist et al., 2014), the entrance area of the proper Baltic Sea. The only other teredinid species known from this area is *Psiloteredo megotara*, but the abundances of this species are low with only a few observations of single individuals (Norman, 1977; Appelqvist et al. 2014). Both of these studies reported the sampling site Mölle in the Kattegat as the southern limit of occurrence of *P. megotara*.

Unlike the short-term outbreaks in the 1930s and 1950s, for at least 20 years a permanent occurrence of *T. navalis* has now been detected at the German coast of the Baltic Sea which is a very new situation for this region. As mass outbreaks are known to be typical for many invasive species immediately after their introduction (Gollasch et al., 2009), the outbreaks of *T. navalis* are in close temporal connection with large salt water intrusions from the North Sea into the Baltic Sea. Matthäus (1993) reported such salt water inflows at the beginning of the 1930s and 1950s and in both decades there have been reports of *T. navalis* in the Baltic Sea. Also, the last major mass outbreak of *T. navalis* was accompanied by a massive salt water intrusion in 1993. Therefore, the documented mass occurrences of *T. navalis* over the last decades in the Baltic Sea may reflect these recurring saltwater inflows and consequently, there could be a link between the mass outbreaks and the various massive saltwater intrusions. As an exception compared to other teredinid species, *T. navalis* even expands into brackish waters which were not inhabited before as seen now for the areas of Rotterdam and Bremerhaven (Tuente et al., 2002; Paalvast and van der Velde, 2011). In contrast, Appelqvist et al. (2014), however, found no indications of a range expansion for the last 35 years (1973–2008) in Swedish waters, where *T. navalis* is well established.

Therefore, the question arose whether the currently found wood-boring bivalve species in this area is still *T. navalis* or perhaps a newly introduced sibling species in the proper Baltic Sea. Nevertheless, the resolution of the conservative 18S as well as the 28S gene is too low to clarify the question whether a sibling species exists in the Baltic Sea or in other marine regions. For this reason, the more variable mitochondrial COI gene was examined, which provides better taxonomic resolution at and below species level. The COI data also confirm that *T. navalis* is only one species, and that no sibling species exist, as the maximum intraspecific pairwise distance is 0.19% and thus, well below the 2% threshold between species (Ratnasingham and Hebert, 2013). The fact, that the combined 18S/28S sequences as well as the COI sequences showed no differentiation between *T. navalis* specimens from Europe and North America, strongly supports the hypothesis of a single species on both sides of the Atlantic Ocean. Although the number of samples

from North America is rather small, the results point to at least an amphi-Atlantic distribution of this species.

Both, in the Baltic as well as in the North Sea, a high COI haplotype diversity was observed for *T. navalis* (Table 3). This is very unusual for invasive species because newly established populations often lose genetic variation due to a founder event (e.g. Thulin and Tegelstrom, 2001; Golani et al., 2007; Tarnowska et al., 2013). Many recent studies working on aquatic invaders use molecular markers to trace the colonization history and to analyse the respective population structure (Roman and Darling, 2007; Tarnowska et al., 2013). Unfortunately, at this point no comparison can be done because no other previously published studies of COI haplotype data of *T. navalis* exists. Therefore, it would be desirable to collect more COI data using the newly provided primers to address the still unknown origin and the colonization history of the Baltic Sea and other European coastal waters in general.

Thus, for a detailed view on the intraspecific relationships within *T. navalis* a median-joining network was calculated (Fig. 3) based on the COI dataset. This network showed no differentiated clades or recognizable lineages as compared to other invertebrates (e.g. Bastrop et al., 1998; Krebes et al., 2011). The most common COI haplotype found for *T. navalis* is represented at all six sampling areas suggesting also an amphi-Atlantic distribution of the species. This dominant haplotype was found in 47.7% of all individuals within the network showing a large difference to the second most common haplotypes representing only 3.5% of the individuals. Furthermore, the ancestral haplotype is the one with the highest frequency and probably also the oldest involved in this network, because there is a direct relationship between haplotype frequencies and the age of haplotypes (Ferreri et al., 2011). Therefore, it will be assumed that the dominant haplotype found in the present study is to be most likely the starting point of all other haplotypes. A star pattern phylogeny (Fig. 3) is indicating an expanding population because new haplotypes mostly arise from the most abundant haplotype. In addition, these star-shaped patterns in phylogenetic networks associated with high haplotype diversity and low nucleotide diversity as shown in Table 3, are often an indication of a recent population expansion in the history of the species (Fratini and Vannini, 2002; Ferreri et al., 2011). The low differentiation and frequency of rare haplotypes also point to a population expansion from a small number of individuals after a genetic bottleneck or a founder event (Allcock and Strugnell, 2012). This is well known for other molluscs too, for instance for the *Mytilus* spp. species complex (Steinert et al., 2012). Regarding the shape of the network, the predicted recent population expansion may also be interpreted as an expansion from a single source (Klicka et al., 2011). This consideration is shared by other authors either in conjunction with a bottleneck effect or with a founder effect, such as a recolonization from a glacial refugium after the last European Ice Age (Allcock and Strugnell, 2012).

Whether the supposed population expansion of *T. navalis* is due to a founder or bottleneck effect, cannot be clarified with the available data from the present study. The finding that all investigated sampling sites share the dominant ancestral haplotype could indicate that all of them represent the source population or the population of origin respectively. At the same time it is also difficult to make reliable statements on the cosmopolitan distribution of *T. navalis* because unfortunately there is a lack of trustworthy sightings around the world regarded to the taxonomic difficulties. Clarification of these still open questions should be

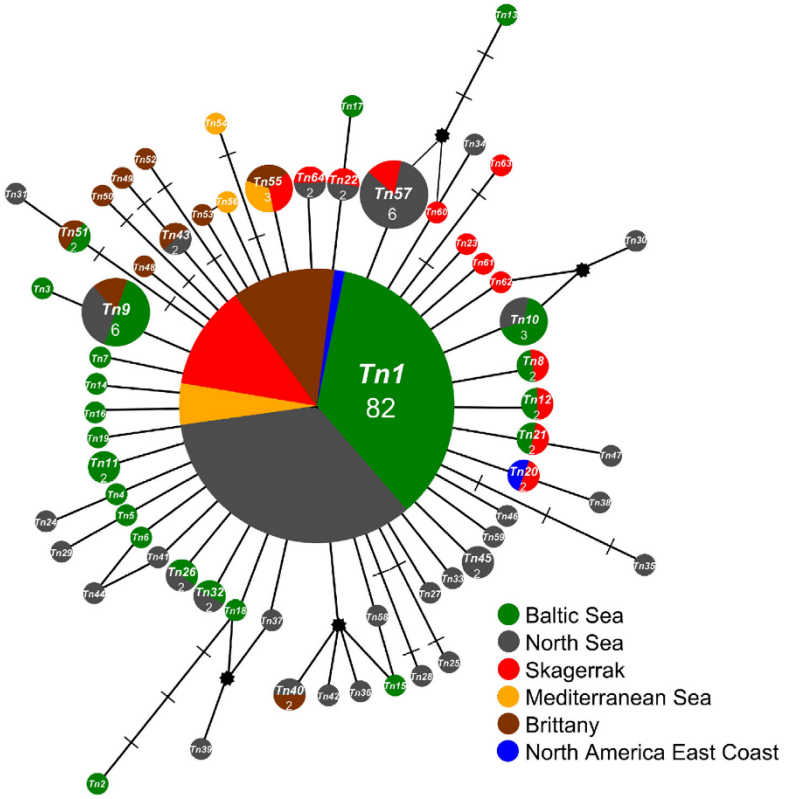

Fig. 3. Haplotype median joining network based on COI analysis. Each circle represents a different haplotype (Tn1–Tn64) and the size is proportional to the number of individuals sharing the same haplotype. Number of sequences included is below the title of the haplotype. If there is none, the number is one. Black stars are median vectors indicating not sampled or missing haplotypes. Short bars show the number of mutation steps between the haplotypes. If there is no bar, the number of mutation steps is one.

an aim for further investigations with a more comprehensive sampling and the inclusion of high resolution nuclear markers for proper population genetic analyses.

5. Conclusions

The taxonomic classification of four European wood-boring molluscs (*T. navalis*, *L. pedicellatus*, *N. norvagica*, *T. dominicensis*) supports the phylogenetic hypothesis of monophyly of the Teredinidae as suggested by previous authors. All data presented and points discussed above suggest that the analysed samples of *T. navalis* from six different areas in Europe and North America represent a single species and no sibling species exist at the studied sampling areas. This confirms the hypothesis that *T. navalis* is a worldwide spread wood-borer with an amphi-Atlantic distribution. Using the barcoding approach, it was verified that *T. navalis* is the only teredinid species in the shallow waters of the proper Baltic Sea. This species seems to expand its recent populations. Whether the occurrence of *T. navalis* in the Baltic Sea is based on a biological invasion caused by human introduction from somewhere around the world or a purely range expansion starting from an autochthonous European *T. navalis* population, cannot be clarified with the present data. A more comprehensive sampling is needed to further determine the place of origin of *T. navalis* and to examine the spreading of *T. navalis* across Europe. The required COI sequences can now be easily obtained with the new designed primers from this study.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2015.11.008.

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3.2 Genetic population structure and demographic history of the widespread common shipworm *Teredo navalis* Linnaeus, 1758 (Mollusca: Bivalvia: Teredinidae) in European waters inferred from mitochondrial COI sequence data

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Genetic Population Structure and Demographic History of the Widespread Common Shipworm *Teredo navalis* Linnaeus 1758 (Mollusca: Bivalvia: Teredinidae) in European Waters Inferred from Mitochondrial COI Sequence Data

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The first documented scientific reports of the common marine shipworm *Teredo navalis* (Bivalvia) for Central European waters date back to the time between 1700 and 1730 in the Netherlands. During the following centuries there were several irregular mass occurrences reported for both the North Sea and the Baltic Sea. These events were accompanied by massive destruction of wooden ships and coastal protection structures. In this study, the first population analysis of *T. navalis* is presented with the aim to detect the genetic population structure in the waters of Central Europe. The mtDNA COI (cytochrome c oxidase subunit I) locus was found as suitable molecular marker and hence a 675 bp gene fragment was studied. A total of 352 *T. navalis* specimens from 13 different sampling sites distributed across Central Europe were examined. Subsequently, various population genetic indices including F_{ST} values and an AMOVA analysis were applied for the description of the population structure. To visualize the distribution of haplotypes at the different sampling sites two median-joining networks were calculated. In addition, the past demographic structure of the *T. navalis* population was analyzed, among others by calculating Tajima's D, Fu's F and the mismatch distribution. Finally, all computations of the population genetic indices could not reveal differentiated populations or any kind of distinct population structure in *T. navalis*. The network analyses revealed "star-like" patterns without differentiated substructures or demes. Therefore, it can be assumed that a sudden expansion of this species took place without any indications of neither a bottleneck nor a founder effect for the study area. The results of this study support the concept of a regional panmictic population in the waters of Central Europe with unhindered migration of individuals (e.g., via pelagic larvae) between the various sampling sites as reflected by a high gene flow.

Keywords: ***Teredo navalis*, teredinidae, bivalvia, genetic diversity, demographic expansion, COI, Baltic Sea, North Sea**

INTRODUCTION

Marine wood-boring Bivalvia are well known in European waters for a long time and according to the latest findings, nine different shipworm species (Teredinidae) are currently known in this region (Borges et al., 2012). They are typically associated with natural and man-made wood such as mangrove roots in tropical regions, coastal protection and harbor facilities as well as floating structures such as wooden ships and driftwood. Since these xylotrophic organisms decompose wood much faster than marine fungi or bacteria (Borges, 2007), they have caused considerable damage in Europe and worldwide over the last centuries (Distel et al., 2011). One of the most widespread and most destructive wood-boring Teredinidae in European waters is the common marine shipworm *Teredo navalis*. Since the origin and natural habitat of *T. navalis* is still unclear this species is currently considered cryptogenic and invasive in European waters (Borges et al., 2014).

A closer look into the literature shows that reports of shipworms from the South of Europe are considerably more abundant and earlier noted than for the North (Redeke, 1912; Moll, 1914). The first descriptions from the Mediterranean Sea date back to the authors of the classical antiquity like Aristoteles, Ovid, and Pliny (Redeke, 1912; Moll, 1914). They mentioned different kinds of shipworms, but the species involved were unknown. The next observations of Teredinidae in the Mediterranean Sea dated considerably later from the sixteenth to seventeenth century (Moll, 1914; Streftaris et al., 2005) and thus coincide with reports from Central Europe (North Sea).

The first credible proofs of *T. navalis* for Central European waters are reported from several authors for the North Sea around 1700–1730, the years of the first documented mass outbreak in the coastal waters of the Netherlands (e.g., Vrolik et al., 1860; Van Benthem Jutting, 1943). All authors assigned the attacks of wooden coastal protection structures unanimously to *T. navalis*. For many non-indigenous marine species mass occurrences typically have been described some years after their introduction (Gollasch et al., 2009), often resulting in their discovery. Also the sample material for the species description of *T. navalis* by Linnaeus in the year 1758, was collected in the Netherlands during this time by Sellius (Redeke, 1912).

For the Baltic Sea the first observations of *T. navalis* were documented from the Bay of Kiel in 1835 (Meyer and Möbius, 1865). The last and ever-present mass occurrence for the Baltic Sea was recorded in 1993 (Sordyl et al., 1998). Since then a stable and self-reproducing population has been established at the southern Baltic Sea coast, as Sordyl et al. (1998) detected free-swimming larvae of *T. navalis* in the water column. The current distribution of this species in the North Sea and Baltic Sea can be considered as stable as reflected by several recent studies (e.g., Paalvast and van der Velde, 2011; Appelqvist et al., 2015a; own unpublished data).

Although, much is still unknown about the ecology and especially the physiology of *T. navalis*, temperature, salinity and water currents determine to a great extent the distribution of this species. Regarding the required abiotic conditions for reproduction and growth, the optimum salinity ranges between 7 and 35 (Blum, 1922; Nair and Saraswathy, 1971) while the optimum water temperature is between 15 and 25°C (Roch, 1932; Kristensen, 1969). *Teredo navalis* is a protandric hermaphrodite species reaching sexual maturity seven to 8 weeks after metamorphosis (Grave, 1942). The larvae are released into the water column ~5 days after fertilization with a high release capacity of up to five million larvae per female and year (Kaestner, 1982). This short maturation period in combination with a high larvae release leads to a high reproductive potential. In combination with a short generation time this could result in about three to four generations per year. For the cold temperate waters in Central Europe, however, only one generation per year is expected for this species (see Section Genetic differentiation of populations).

Little is known so far whether *T. navalis* was absent between the mentioned mass occurrences or survived in reduced populations. This leads to the question whether the reported periodic mass occurrences of *T. navalis* in the North Sea and the Baltic Sea during the nineteenth and twentieth century, are reflected in their current distribution and population structure. Therefore, in the present study it was evaluated, whether and to what degree *T. navalis* is genetically structured. The conceivable existence of a self-containing Baltic Sea population separated from a North Sea/Atlantic Ocean population was a second question to be addressed. Furthermore, a third aim was to assess the possibility of a demographic expansion of *T. navalis* in the past together with the attempt to determine the point of origin of this expansion in time.

The COI (cytochrome c oxidase subunit I) locus has been proven to be a useful marker in population genetics and was employed in many studies for species analyses of the Bivalvia (e.g., Sirna Terranova et al., 2006; Sá-Pinto et al., 2012; Vergara-Chen et al., 2013). While in a previous study specific COI primers were provided for the first time and different regions in Europe and North America were investigated with respect to species identification, Weigelt et al. (2016) showed that no sibling species of *T. navalis* exist in the North Atlantic and the adjacent European seas. The present study investigated *T. navalis* in Central Europe marine waters at the population genetic level. Therefore, the genetic diversity of 352 *T. navalis* specimens collected along a salinity gradient was determined by comparing different sampling sites from Brittany in the west to the southern Baltic Sea in the east.

MATERIALS AND METHODS

Sampling and Storage

A total number of 352 specimens of *T. navalis* was analyzed. Collecting took place during two time periods between 1999–2000 and 2011–2016 at 13 different sampling sites in Central Europe marine waters (**Figure 1**). A complete list of all specimens examined in this study as well as an overview of all sampling sites and the corresponding GPS coordinates are given in Table S1 in the supplementary data.

The specimens were collected from submerged permanent sources like wooden coastal protection structures and from

deployed test panels which consisted of non-durable pine wood (*Pinus sylvestris*) suitable for sampling teredinids (DIN EN 275, 1992; Borges et al., 2014). The taxonomic nomenclature is according to Turner (1966, 1971). Specimens from 1999 to 2000 were stored frozen at −60°C and later transferred into 96% ethanol. All other specimens were directly stored in 96% ethanol prior to further DNA analysis.

DNA Extraction, Amplification, and Sequencing

The DNA was extracted from mantle and muscle tissue by using "innuPREP Forensic Kit" (Analytik Jena) following protocol five of the user manual and the final elution volume was 200 µl. The mitochondrial COI gene fragment was amplified using polymerase chain reaction (PCR) performed with different primer pairs. The PCR amplification parameters and the different combinations of primers applied are shown in **Table 1**. All PCR reactions were performed with Analytik Jena FlexCycler in a 30 µl reaction volume consisting of 10x PCR buffer, 4.0 mM $MgCl_2$, 0.75 U Moltaq (Molzym GmbH & Co.KG), 62.5 µM of each dNTP, 1 pmol of each COI primer and 3 µl DNA template. Thereafter, 5 µl of each PCR product were visualized under UV-light in 1% agarose gel and checked manually. PCR products were purified with "innuPREP Gel Extraction Kit" (Analytik Jena) following the manufacturer protocol and then used for sequencing. The sequencing of PCR products was performed using dideoxy chain termination method and cycle sequencing (Sanger et al., 1977) using "BigDye® Terminator v.1.1 Cycle Sequencing Kit" (Applied Biosystems). The primers used for sequencing were the same as those for PCR amplification. Sequencing products were purified following the GenomeLab Sequencing Chemistry Protocol 3.2 (Beckman Coulter).

The cycle sequencing products were analyzed by using capillary separation on an Applied Biosystems Genetic Analyzer 3130*xl* (Hitachi) and were sequenced in both directions. All sequences obtained in this study were deposited to NCBI GenBank. For detailed information about all sequences determined, the GenBank accession numbers (MF071107–MF071198) and a complete list of all haplotypes, see Table S1 in the supplementary data.

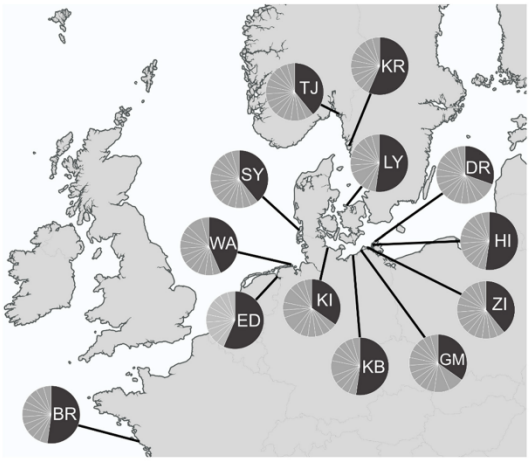

FIGURE 1 | Sampling sites with COI haplotypes frequencies per sampling site. Dark gray: the most common haplotype Tn1, light gray: all other haplotypes. BR, Brittany; ED, Emden; WA, Wangerooge; SY, Sylt; TJ, Tjärnö; KR, Kristineberg; LY, Lynaes; KI, Kiel; KB, Kühlungsborn; GM, Graal-Müritz; ZI, Zingst; HI, Hiddensee; DR, Dranske.

Phylogenetic Analyses

Recorded COI sequences were manually checked and aligned with BioEdit (Hall, 1999) using the ClustalW (Thompson et al., 1994) algorithm. The common population indices such as number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), the number of nucleotide differences (k) plus their corresponding standard deviations were obtained from all 13 sampling sites using the program DnaSP (Librado and Rozas, 2009). Phylogenetic analyses were performed using MEGA6 (Tamura et al., 2013) and PopArt 1.7 (http://popart.otago.ac.nz). For calculating the two phylogenetic networks the median-joining method (Bandelt et al., 1999) implemented in PopArt 1.7 was used.

Genetic Differentiation of Populations

For the different calculations of the genetic population structure a first dataset (DS 1) was created. From each of the 13 sampling sites 23 specimens (DS 1, $n = 299$) were taken for analysis to avoid under-sampling due to small sample sizes. For a second dataset (DS 2) specimens from a small scale sampling area between the two sampling sites Kühlungsborn and Zingst were pooled.

TABLE 1 | PCR parameter.

Gen	Primer name	Primer sequence (5′ → 3′)	Sense	References
COI	Ter-fw II[1]	GTTTCTGGTTAATGCCTAGATC	forward	Weigelt et al., 2016
	Ter-fw III[2]	GG(A/G/T)GATAT(A/G)GC(T/C)TT(T/C)CCTCG	forward	Weigelt et al., 2016
	Ter-rev I[1,2]	GCTCTGGATACGACAATCCCAG	reverse	Weigelt et al., 2016
	Mat rev[2]	AT(A/T/C/G)GC(A/G)AA(A/T/C/G)AC(A/T/C/G)GC(A/T/C/G)CC(T/C)AT	reverse	Matsumoto, 2003
Amplification parameter	initial denaturation 94°C, 2 min; 37 cycles: 94°C for 20 s, 52°C for 20 s, 72°C for 60 s; final extension 72°C, 5 min			

Primers used for PCR as well as for sequencing and PCR amplification parameters for amplification of the mitochondrial COI gene. (same superscript numbers denotes PCR primer pairs).

From each of the two time periods 1999–2000 and 2011–2015 36 specimens (DS 2, $n = 72$) were taken for temporal analysis. 19 specimens belong to both data sets (for details see Table S1).

The comparisons between the different sampling sites for DS 1 were performed calculating population pairwise F_{ST} values as implemented in Arlequin 3.5.1.3 (Excoffier and Lischer, 2010). The significance of the F_{ST} values was tested with 10,000 permutations. Hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) was performed to test the genetic variance of two pre-defined groups: North Sea and Baltic Sea. The level of significance was also determined by means of 10,000 permutations.

To test for effects of past demographic events, like e.g., bottleneck effects or population expansions, the null hypothesis of neutral evolution of the COI locus was tested using Tajima's D test (Tajima, 1989) and Fu's *F* test (Fu, 1997) as implemented in Arlequin 3.5.1.3. The significance of both statistical tests was evaluated by 10,000 replicates. Moreover, Ramos-Onsins and Rozas" R_2 value (Ramos-Onsins and Rozas, 2002) was calculated since it is more stable for small sample sizes. The statistical significance was checked with 1,000 permutations. The latter calculation was done with DnaSP (Librado and Rozas, 2009).

To examine the possibility of a past demographic expansion a mismatch analysis by examination of frequency distributions of pairwise differences between sequences for DS 1 was done (Rogers and Harpending, 1992). The distribution is multimodal for populations at a demographic equilibrium, but unimodal for populations during a recent demographic or range expansion (Slatkin and Hudson, 1991; Rogers and Harpending, 1992; Excoffier, 2003). The sum of square deviations (SSD) between two mismatch distributions was also calculated with Arlequin 3.5.1.3.

The associated mismatch parameter Tau (τ) was used to estimate the time elapsed since the expected expansion started in the past. According to the equation $t = \tau/2u$, the mutation rate (u) for the gene under study was needed. In the absence of a specific substitution rate for the COI gene for *T. navalis*, two different molluscan specific substitution rates of 1.0% per million years (Strasser and Barber, 2009) ($u = 6.75 * 10^{-6}$) and 2.0% per million years and site (Layton et al., 2014) ($u = 1.35 * 10^{-5}$) were used. The generation time adopted was one year for Bivalvia in cold temperate waters (based on own unpublished data for *T. navalis*).

RESULTS

Genetic Diversity

The COI locus provided reproducible results and hence was used for the aims of the present study. In a first step, the 299 COI sequences from the 13 sampling sites of DS 1 were taken to calculate the various population indices. The population genetic indices such as the total number of haplotypes, the haplotype diversity, the nucleotide diversity and the number of average nucleotide differences for all sampling sites are shown in **Table 2**.

The DS 1 contained 114 mutations with 111 segregating sites resulting in 113 different haplotypes. The Hd for the whole DS 1 was 0.776. The highest haplotype diversities were found at the most eastern sampling site Dranske (Baltic Sea) (0.913), followed by Kiel and Graal-Müritz (both Baltic Sea) (0.881) and Sylt (North Sea), Tjärnö (Skagerrak) and Zingst (Baltic Sea) (0.858). The lowest haplotype diversities were determined for the sampling sites Emden (Ems estuary, North Sea) and Kristineberg (Skagerrak) (0.692). The nucleotide diversity showed similar trends with the highest diversities found at the sampling sites Kiel (0.00327) and Dranske (0.00317) and the lowest at the sampling site Kristineberg (0.00142).

TABLE 2 | Population genetic indices for all sampling sites.

Sampling site	N	S	H	Hd (SD)	π (SD)	K
Brittany^{NS}	23	14	12 (6)	0.739 (0.0101)	0.00192 (0.00042)	1.296
Emden^{NS}	23	17	11 (4)	0.692 (0.0109)	0.00219 (0.00058)	1.478
Wangerooge^{NS}	23	14	13 (4)	0.818 (0.082)	0.00192 (0.00036)	1.296
Sylt^{NS}	23	18	15 (11)	0.858 ((0.073)	0.00244 (0.00046)	1.644
Tjärnö^{NS}	23	16	15 (5)	0.858 (0.0109)	0.00206 (0.00033)	1.391
Kristineberg^{NS}	23	11	11 (6)	0.692 (0.073)	0.00142 (0.00035)	0.957
Lynaes^{B}	23	12	11 (7)	0.735 (0.100)	0.00166 (0.00036)	1.123
Kiel^{BS}	23	19	14 (4)	0.881 (0.062)	0.00327 (0.00060)	2.206
Kühlungsborn^{BS}	23	16	11 (5)	0.735 (0.100)	0.00230 (0.00057)	1.549
Graal-Müritz^{BS}	23	17	14 (10)	0.881 (0.062)	0.00242 (0.00040)	1.636
Zingst^{BS}	23	22	15 (10)	0.858 (0.073)	0.00283 (0.00054)	1.913
Hiddensee^{BS}	23	15	12 (5)	0.739 (0.104)	0.00227 (0.00056)	1.534
Dranske^{BS}	23	21	16 (5)	0.913 (0.052)	0.00317 (0.00057)	2.142
North Sea (median)	138	90	77 (36)	0.779 (0.092)	0.00199 (0.00039)	1.344
Baltic Sea (median)	138	110	82 (39)	0.870 (0.068)	0.00263 (0.00057)	1.775
Total	299	111	113 (82)	0.776 (0.025)	0.00220 (0.00014)	1.488

N, number of specimens; S, segregating sites; H, number of haplotypes (unique haplotypes in brackets); Hd, haplotype diversity (with standard deviation); π, nucleotide diversity (with standard deviation); K, number of average nucleotide differences; NS, North Sea; B, Belt Sea; BS, Baltic Sea. Sampling sites are listed from west to east.

All genetic diversity values including standard deviations as well as the number of nucleotide differences (k) are given in **Table 2**.

The analyses of DS 2, which contained 72 COI sequences, resulted in 39 segregating sites with 31 haplotypes. The total Hd for DS 2 was calculated as 0.775, which is almost identical to DS 1 (0.776).

Genetic Population Structure

A median-joining network was calculated for each of the two datasets (DS 1 and DS 2). Both of them showed a "star-like" phylogeny with one predominant central haplotype (**Figures 2**, **3**). The haplotype network of DS 1 (**Figure 2**) was subdivided into the most abundant central haplotype Tn1, which included 134 sequences, and the remaining 112 haplotypes with one to six sequences each. The central haplotype Tn1 is represented in 44.8% of all specimens, whereas another 30 haplotypes are shared by more than one individual (27.4%). For Tn1 the highest frequency was found at Emden (Ems estuary, North Sea) and Kristineberg (Skagerrak) (56.5%) and the lowest at Kiel (Baltic Sea) (30.4%). The different haplotype frequencies for all sampling sites are shown in **Figure 1**. The highest absolute number of haplotypes was determined at Dranske (Baltic Sea) (16) followed by Sylt (North Sea) and Tjärnö (Skagerrak) (15), the lowest numbers for Emden (Ems estuary, North Sea), Kristineberg (Skagerrak), Lynaes and Kühlungsborn (all Baltic Sea) (11).

82 unique haplotypes (27.8% of DS 1) occurred only at one respective sampling site. These unique haplotypes constituted the largest proportion (72.6%) of haplotypes to the network and were three or less mutation steps distinct from Tn1. On the other hand 31 haplotypes were found at more than one

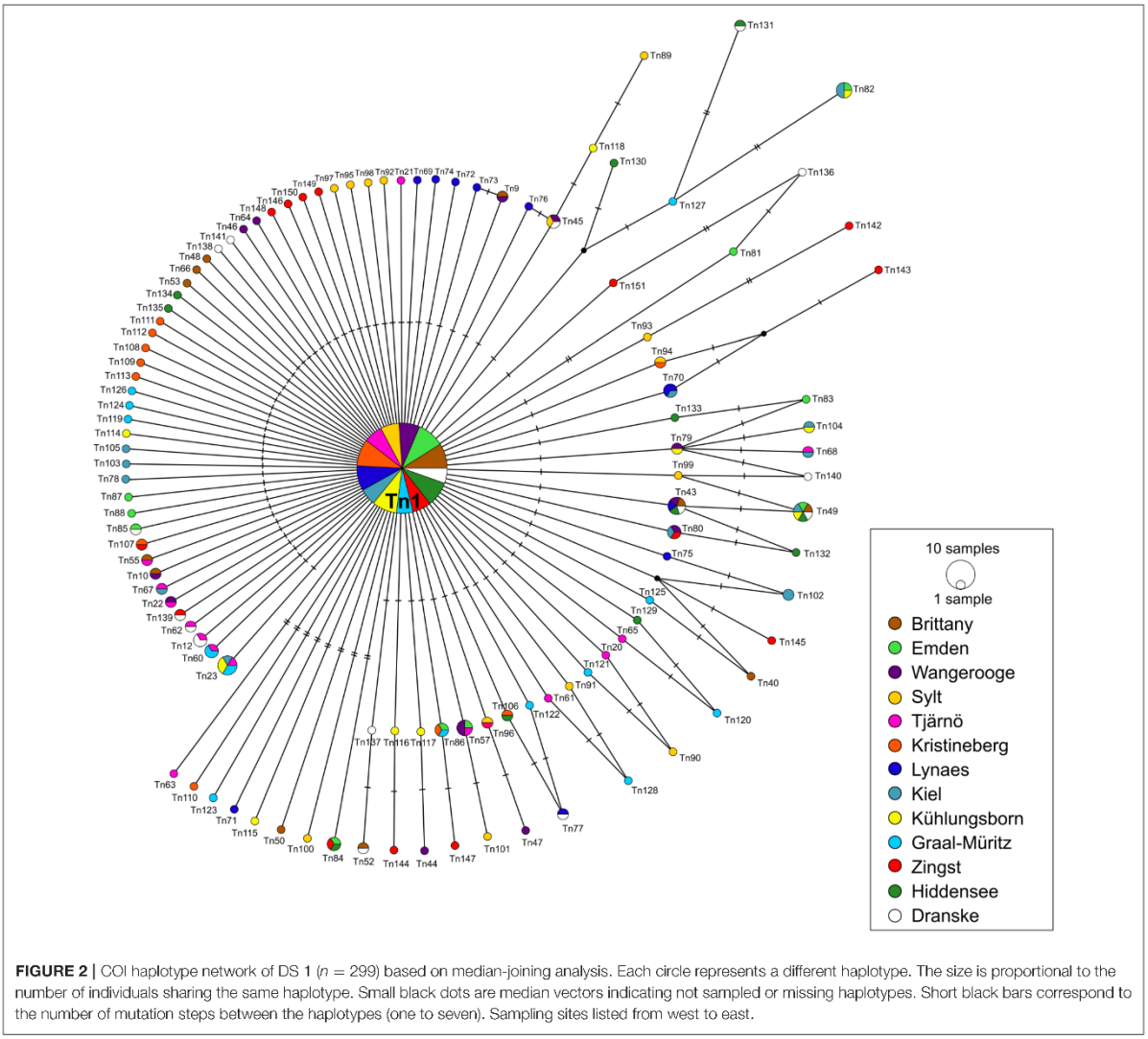

FIGURE 2 | COI haplotype network of DS 1 (n = 299) based on median-joining analysis. Each circle represents a different haplotype. The size is proportional to the number of individuals sharing the same haplotype. Small black dots are median vectors indicating not sampled or missing haplotypes. Short black bars correspond to the number of mutation steps between the haplotypes (one to seven). Sampling sites listed from west to east.

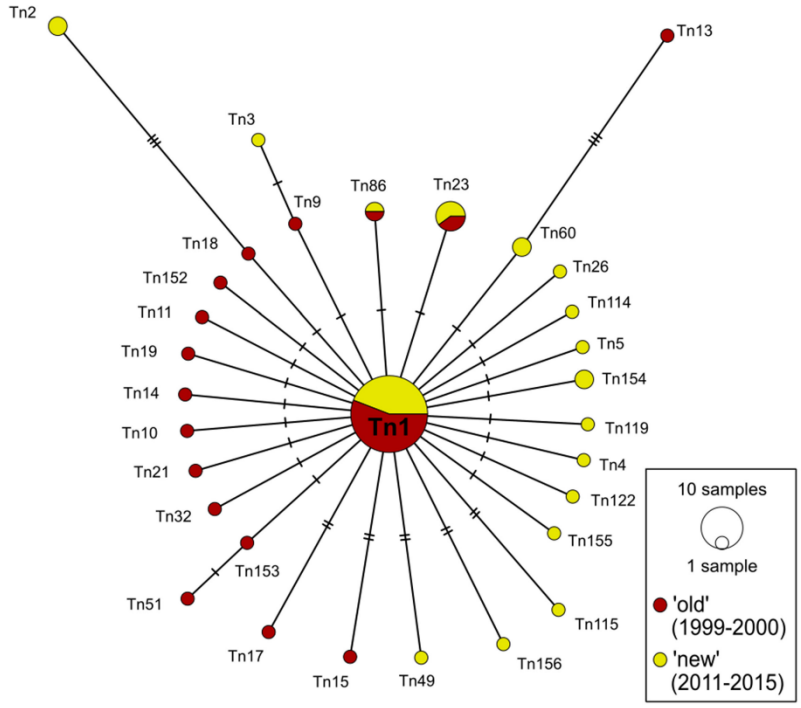

FIGURE 3 | COI haplotype network of DS 2 ($n = 72$) based on median-joining analysis. Each circle represents a different haplotype. The size is proportional to the number of individuals sharing the same haplotype. Small black dots are median vectors indicating not sampled or missing haplotypes. Short black bars correspond to the number of mutation steps between the haplotypes (one to eight).

sampling site, which corresponded to a share of 27.4%. The highest percentage of unique haplotypes per sampling site was determined at Sylt (North Sea) (73.3%), the lowest for Kiel (Baltic Sea) (28.6%). The largest genetic distance between two haplotypes of the median-joining network was seven mutation steps (**Figure 2**).

As described above, the 72 specimens for DS 2 were collected during two periods from 1999 to 2000 ("old") and from 2011 to 2015 ("new"). The total of 31 different haplotypes in this network splitted up into three shared haplotypes determined for both time periods and in additional 14 haplotypes each found only in one of the two time periods (**Figure 3**). Unique haplotypes contributed with 90.4% to the whole network. The different haplotypes of DS 2 formed, similarly to DS 1, a "star-like" network with the dominant haplotype Tn1 in the center. This ancestral haplotype represented 50% of all specimens ($n = 36$) included in the network, which was divided with a ratio of 55.6%: 44.4% in "old" ($n = 20$) and "new" ($n = 16$) sequences, respectively. The other 36 specimens distributed to one haplotype consisting of three sequences ($n = 3$), four haplotypes of two sequences ($n = 8$) and finally 25 sequences which were represented by only one individual specimen.

As shown in **Tables 3, 4** none of the more sensitive values for the evaluation of population structures (F_{ST} and AMOVA) showed a significant population differentiation for the area under investigation. The pairwise F_{ST} values ranged from −0.0150 to 0.0172, and were not significant at any sampling site (**Table 3**). According to Wright (1978) such values indicate a very low differentiation between the sampling sites.

The hierarchical AMOVA analysis revealed a low value of 0.12% for the genetic variation among the two groups that have been sampled (North Sea and Baltic Sea). For the differentiation of populations (= sampling sites) within the two groups a negative value of −0.08 was calculated. The highest value of variation (99.96%) was calculated for the genetic variation within the populations (= sampling sites). All three values were not significant (**Table 4**).

Genetic Inferences of Historical Demography

Tajima's D and Fu's *F* test yielded negative and highly significant values (**Table 5**) for DS 1 indicating a recent demographic population expansion (Tajima, 1989; Fu, 1997). This is supported also by the R_2 statistics which was significant as well (**Table 5**). To detect population growth the R_2 test is more sensitive for small sample sizes (Ramos-Onsins and Rozas, 2002).

The mismatch distribution of DS 1 is closely fitted to a model of sudden expansion (**Figure 4**). The corresponding graph of the

TABLE 3 | F_{ST} values (pairwise differences) for the different sampling sites included in DS 1.

	BR^{NS}	ED^{NS}	WA^{NS}	SY^{NS}	TJ^{NS}	KR^{NS}	LY^{NS}	KI^{NS}	KB^{NS}	GM^{NS}	ZI^{NS}	HI^{NS}	DR^{NS}
BR^{NS}	–												
ED^{NS}	−0.0054	–											
WA^{NS}	−0.0056	−0.0080	–										
SY^{NS}	0.0028	−0.0020	−0.0075	–									
TJ^{NS}	0.0001	−0.0050	−0.0084	−0.002	–								
KR^{NS}	0.0035	−0.0030	0.0035	0.0001	0.0000	–							
LY^{B}	−0.0013	0.0001	0.0003	0.0030	0.0031	0.0002	–						
KI^{BS}	0.0072	−0.0100	0.0072	0.0123	0.0049	0.0172	0.0076	–					
KB^{BS}	0.0004	−0.0150	−0.0050	−0.0020	−0.0050	0.0063	0.0046	−0.0160	–				
GM^{BS}	0.0055	−0.0020	0.0080	0.0026	−0.0100	0.0032	0.0058	0.0067	−0.0040	–			
ZI^{BS}	−0.0011	−0.0090	0.0001	−0.002	−0.0010	−0.0080	−0.0020	0.0024	0.0002	0.0002	–		
HI^{BS}	−0.0048	−0.0150	0.0033	0.0100	0.0107	0.0096	0.0035	0.0010	−0.0020	0.0054	−0.0020	–	
DR^{BS}	−0.0084	−0.0120	0.0027	0.0024	0.0004	0.0077	0.0017	0.0026	−0.0060	0.0065	0.0022	−0.0120	–

BR, Brittany^{NS}; ED, Emden^{NS}; WA, Wangerooge^{NS}; SY, Sylt^{NS}; TJ, Tjärnö^{NS}; KR, Kristineberg^{NS}; LY, Lynaes^{B}; KI, Kiel^{BS}; KB, Kühlungsborn^{BS}; GM, Graal-Müritz^{BS}; ZI, Zingst^{BS}; HI, Hiddensee^{BS}; DR, Dranske^{BS}. NS, North Sea; B, Belt; BS, Baltic Sea.

TABLE 4 | Values of hierarchical AMOVA analysis of DS 1.

	d.f.	% of variation	F-statistics	*p*-Values
Source of variation				
Among groups	1	0.12	$F_{CT} = 0.00125$	0.1634^{ns}
Among populations within groups	11	−0.08	$F_{SC} = -0.0008$	0.6458^{ns}
Within populations	286	99.96	$F_{ST} = 0.00042$	0.5153^{ns}

The genetic structure of the 13 sampling sites (in this case sampling site = population) included in two groups (North Sea and Baltic Sea) ($p < 0.05$, ns, not significant; d.f., degrees of freedom) was tested.

TABLE 5 | Results of neutrality tests of demographic parameters for dataset 1.

Statistical test	Value	Significance *p*
Tajima's D	**−2.7526**	0.00*
Fu's F	**−27.5720**	0.00**
R_2 statistic	**0.006636**	0.00*
SSD	0.0006^{ns}	0.4276*

Significant ($p < 0.05$, $p < 0.02$**, ns, not significant) values are indicated in bold type. (SSD, sum of square deviations).*

mismatch distribution for the sudden expansion model showed a unimodal curve progression indicating a demographic expansion of *T. navalis* in the past. The corresponding SSD-value (**Table 5**) is not significant which reveals that the population is still in an expansion phase and not at equilibrium (Schneider and Excoffier, 1999).

With the associated τ-value of 1.5 for DS 1 and the different substitution rates of 1.0 and 2.0% for Bivalvia a time span for the beginning of the expansion was calculated. Assuming a mutation rate between $6.75 * 10^{-6}$ and $1.35 * 10^{-5}$ for the COI gene per generation and a generation time of one per year, the hypothetical beginning of the expansion took place ∼55,556–111,111 years ago.

DISCUSSION

In a previous study specific COI primers for *T. navalis* were introduced and COI sequence data were obtained for the first time of molecular species identification on samples collected in different regions in Europe and North America (Weigelt et al., 2016). In the present study the main goal was to evaluate the population structure of *T. navalis* in Central European waters.

The two calculated median-joining networks showed a "star-like" phylogeny and the frequency of the central haplotype (Tn1) was dominating the complete network in both cases. The shared haplotypes in DS 1 (27.4%) are arbitrarily distributed over all locations and without clear geographic distribution pattern, mainly because all sampling sites were represented in the central haplotype (**Figure 2**). The proportion of three shared haplotypes in DS 2 (9.6% of all haplotypes) for a small scale sampling area between the two sampling sites Kühlungsborn and Zingst (∼100 km apart) over the time scale of 16 years appeared to be low. This might be explained by the loss of haplotypes through genetic drift or possibly rather the gain of new haplotypes in this area by gene flow. The proportion of unique haplotypes in both networks are 72.6% (DS 1) and 80.7% (DS 2), respectively. These comparatively high proportions of unique haplotypes of both networks in relation to all haplotypes can be interpreted as one indicator for an expanding population (Slatkin and Hudson, 1991; Fu, 1997). It is most likely that all of these haplotypes originated from the predominant ancestral haplotype Tn1 after the expected sudden population expansion. Such a pattern and interpretation has been reported before for other marine bivalves, such as *Mya arenaria* (Lasota et al., 2016).

Some unique haplotypes from the initial study (Weigelt et al., 2016) are no longer unique since in the present study they could also been recorded at different sampling sites. This could be an

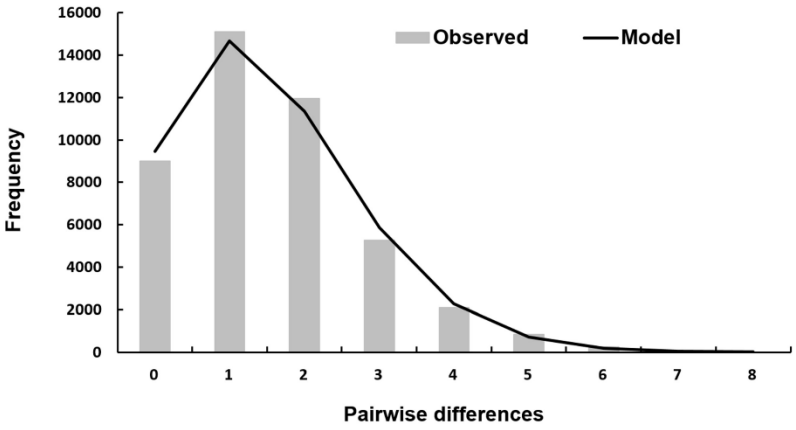

FIGURE 4 | Mismatch distribution of DS 1. Observed pairwise differences between 299 sequences for DS 1 (bars) and the expected mismatch distributions under the sudden expansion model (solid line).

indication for a high genetic diversity which might be reflected by high abundances. Up to 700 animals per dm^2 have been observed in pine wood panels during the investigation period (own unpublished data), which is quite high compared to other regions like the Kattegat (Appelqvist et al., 2015b). Moreover, the network analyses of both datasets revealed no differentiated substructures or demes. These results corresponded with findings from a previous study (Weigelt et al., 2016) in which the determined shape of the network was almost identical.

Since the network analyses displayed no signs of a population structure, the genetic diversity of the different sampling sites was investigated. The haplotype diversity ranged from 0.692 to 0.913 which are common values for Bivalvia (e.g., Mao et al., 2011; Li et al., 2015). Thus, no decrease of the haplotype diversity toward the eastern distribution border of *T. navalis* along the salinity gradient in the Baltic Sea could be demonstrated. Even the easternmost sampling site Dranske (Rügen) showed the highest haplotype diversity and the second highest nucleotide diversity of all sampling sites. At this point it can be summarized, that indications for a founder effect, a bottleneck effect or a generally reduced genetic diversity at the edge of the distribution of *T. navalis* in the Baltic Sea could not be confirmed by the present study.

Also the calculated pairwise F_{ST} values showed no differentiation between the 13 sampling sites since the values ranged only from −0.0150 to 0.0172 for DS 1. These are common F_{ST} values for Bivalvia, since Lasota et al. (2004) found for *Mya arenaria* average F_{ST} values of 0.01 to 0.03 between populations which were hundreds of kilometers apart. Following Wright (1978), F_{ST} values from −1 to 0.05 indicate only a very slight genetic differentiation between populations. Negative F_{ST} values have to be considered as zero values which means that there is no genetic subdivision between the sampling sites especially as none of the values of the present study were significant. Therefore, it is not possible to identify a population structure for *T. navalis* by the calculated F_{ST} values of pairwise differences. Likewise, no differentiation in the population structure can be detected by the AMOVA analysis in DS 1 since almost 100% of the variation are within the sampling sites and not among them (**Table 4**).

All data presented in this study clearly indicate a lack of genetically differentiated populations in *T. navalis* between Brittany and the Baltic Sea, and hence might be interpreted as a regional European panmictic population, which, by definition, assumes that there exists no mating restrictions upon this population. The theoretical framework for a panmictic population of *T. navalis* is, however, not highly probable, because an unrestricted mating between the sessile adults from Brittany to the Baltic Sea along a coastline of ca. 3,900 km seems not to be possible. In addition, water exchange between the North Sea and Baltic Sea is restricted, which also hampers unrestricted mating. Otherwise no biogeographical break could be estimated between the highly saline North Sea and the brackish Baltic Sea and a panmictic population structure of *T. navalis* can be observed. Nonetheless, the determined panmixia for *T. navalis* stands in contrast to recent studies on other marine Bivalvia in the North Sea and Baltic Sea which always displayed a sharp differentiation in the genetic population structure between inside and outside the Baltic Sea (Johannesson and André, 2006; Johannesson et al., 2011). In comparison, the two median values of the haplotype as well as the nucleotide diversity of the Baltic Sea are slightly larger than those from the North Sea (**Table 2**). In this context, the results of the present study of *T. navalis* cannot confirm the findings of a species-by-species meta-analysis of Johannesson and André (2006). These authors showed that a majority of Baltic Sea populations lost genetic diversity compared to populations of the same species in the Atlantic Ocean and adjacent waters. The only exception found in their meta-analysis was *Macoma balthica* which was 30% more diverse inside the Baltic Sea than outside because of two genetically distinct lineages (Johannesson and André, 2006), an Atlantic lineage in the western and a Pacific

lineage in the eastern part of the Baltic Sea (Nikula et al., 2008). However, the occurrence of different genetic lineages can be excluded for *T. navalis* (Weigelt et al., 2016).

Since all sampling sites were not differentiated from each other, the sites seem to be connected by a high gene flow. As restricted gene flow has been reported to cause genetic population structuring (Sá-Pinto et al., 2012), in the present study unrestricted gene flow seems to be still ongoing as no genetic population structure has been determined in *T. navalis*. Also for other marine invertebrates with a high dispersal potential and high abundances such as *Holothuria polii*, unrestricted gene flow is followed by a low population genetic structure over small spatial scales (Vergara-Chen et al., 2010). High dispersal potential and high gene flow of marine invertebrates favors genetic homogenization of populations over broad geographical distances (Maltagliati et al., 2010). Genetic homogeneity and a limited genetic structure has also been observed in other bivalve species, for instance in the invasive soft-shell clam *Mya arenaria* (Lasota et al., 2004; Strasser and Barber, 2009).

The observation that all sampling sites hardly differ genetically is a strong indication that all animals originate from only one source population, which is still unknown. Since the first reports on the occurrence of *T. navalis* in Central Europe date back to the years 1700–1730, the first settlement of this species has presumably be taken place at the coast of the Netherlands. This nation was one of the world's leading global trading nations at that time. Since all other reports on *T. navalis* from this area were mentioned later, a successive range expansion from this starting point in the North Sea to the adjacent waters is the most likely explanation. At this point, the life history strategy of *T. navalis* is helpful as it is very effective during the successful colonization of new patchy habitats (Borges et al., 2014) and the associated further spread into new areas. Due to the pelagic larval stadium a range expansion into the Baltic Sea via the Skagerrak, the Kattegat and through the Danish Straits seems very likely. Natural range expansions from the North Sea into the Baltic Sea are well known from other marine organisms, e.g. for the dinoflagellate *Prorocentrum minimum* (Hajdu et al., 2000) or the polychaete *Marenzelleria viridis* (Essink and Kleef, 1993; Blank et al., 2008).

The importance of oceanographic connectivity for the population structure of marine invertebrates with pelagic larval stages was documented by several authors (e.g., Seebens et al., 2013; Wrange et al., 2016). Since populations of marine bivalves are often characterized by high gene flow among different sampling sites, the absence of a population structure over hundreds of kilometers is not uncommon (Vierna et al., 2012, and references therein). It seems that oceanographic connectivity explains regional genetic structure, rather than geographic distance (Wrange et al., 2016), especially in the context with the dispersal potential of bivalve larvae. As previously mentioned, the short generation time in combination with the high output of larvae and a pelagic lifestyle of up to 34 days in the water column (Nair and Saraswathy, 1971) could lead to a wide spreading via water currents. However, this would not explain a continuous exchange of larvae between the most distant regions investigated in this study (Brittany vs. Baltic Sea). Appelqvist et al. (2015a) simulated in a biophysical model a maximum spreading of *T. navalis* larvae by about 400 km for four different locations in the southern Baltic Sea. In addition, there are also temporal warm water inflows of sea surface water documented for the Baltic Sea, particularly in summer such as in July 2002 and 2003 (Feistel et al., 2004). Such irregular water masses could act as vector for an increased input of *T. navalis* larvae from the North Sea to the Baltic Sea.

Theoretically, also anthropogenic activities via ballast water transport might contribute to a steady distribution of *T. navalis* larvae between different biogeographic regions. Previous studies on marine invertebrates have proven that long distance dispersal can be enhanced by human-mediated vectors (Wrange et al., 2016) and hence have a great influence on the biogeographic distribution and finally on the population genetic structure of a species. Additionally, the location of all sampling sites in an area with a high transport ship traffic (e.g., the area of the Kadetrinne in the Baltic Sea is on average passed by 50,000 ships every year; WSA, 2014) and the previously mentioned presence of *T. navalis* larvae in ballast water (Gollasch, 2002) may play a very important role in the distribution of teredinid species (Borges et al., 2014). A spread of larvae via ballast water also against the main prevailing currents (Seebens et al., 2013) might be responsible to transport larvae across biogeographical boundaries (Canales-Aguirre et al., 2015), which finally favors an increased gene flow. In the light of our data, however, a range expansion from the North Sea into the Baltic Sea by the prevailing water currents is the most likely explanation for the observed population genetic structure of *T. navalis* in both the North Sea and the Baltic Sea.

In the subsequent analyses of the demographic history, the entire dataset (DS 1) was treated as one panmictic population. Fragmentation of habitats in the past may cause population structuring e.g., in form of a genetic bottleneck followed by a sudden demographic expansion (Strasser and Barber, 2009; Lasota et al., 2016). As stated before all analysis of the historical demographic situation of *T. navalis* came to the same result indicating a sudden population expansion after a historical decrease event. The significant negative values of Tajima's D and Fu's *F* test as well as the significant R_2 statistics (**Table 5**) support this assumption (Maltagliati et al., 2010). Also the data of the mismatch distribution analysis support these result. With the determined τ-value (1.5) in combination with the different substitution rates of 1.0 and 2.0% per million years for the COI gene and the assumed generation time of one per year, the calculated starting point for the sudden expansion was ∼55,556–111,111 years ago. The geographical starting point of the expansion of *T. navalis* cannot be determined with the data of this study, but is unlikely to be located in the Central Europe marine waters due to the last glaciation of Europe. The last great glaciation in Europe ended ∼10,000 years before present. At that time parts of the North Sea and the whole Baltic Sea were covered by glaciers (e.g., Grunewald and Scheithauer, 2010). Thus, they could not act as a refugium for a surviving population of *T. navalis* since all marine life was extinguished at that time. As some authors even assume a glacial ice cover south to the Iberian Peninsula (Grunewald and Scheithauer, 2010), the hypothetical territorial starting point of this sudden expansion, if it has been

in Europe, could only be in the Mediterranean Sea or south-west of the Iberian Peninsula. However, the place of origin cannot be verified with the present data set. Therefore, the status of *T. navalis* should still remain cryptogenic as defined by Carlton (1996).

In conclusion, this study shows a high level of genetic diversity and gene flow in the investigated area for the examined mtDNA COI gene. This is reflected by the presence of a regional panmictic population of *T. navalis* in the waters of Central Europe. The lack of any genetic population structure indicates for the expansion history only one source population. Nevertheless, in case of *T. navalis* it seems that the use of this marker is not suitable to answer all questions of the population structure and the expansion history. For a deeper understanding of the colonization history of European marine waters by *T. navalis*, the use of highly polymorphic markers such as e.g. microsatellites would be recommended for future investigation.

AUTHOR CONTRIBUTIONS

RW, HL, and RB collected the samples. RW and RB acquired the sequence data and all authors analyzed and interpreted the data. RW drafted the work and all authors critically revised the work and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmars.2017.00196/full#supplementary-material

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3.3 *Teredo navalis* in the Baltic Sea: larval dynamics of an invasive wood-boring bivalve at the edge of its distribution

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Teredo navalis in the Baltic Sea: Larval Dynamics of an Invasive Wood-Boring Bivalve at the Edge of Its Distribution

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Wooden groin systems on the southwestern Baltic Sea coast are a traditional and important coastal-protection facility, but have been regularly infested and destroyed by the wood-boring bivalve *Teredo navalis* since the early 1990s. The occurrence of *T. navalis* was presumed to be limited mainly by the prevailing low salinities. Recently, a possible range expansion of this invasive species to the more eastern parts of the Baltic Sea has been discussed. *T. navalis* larval settlement was therefore monitored at the distribution boundary of the species in the Baltic Sea over a period of 4 years. At 7 stations along the prevailing salinity gradient on the Mecklenburg-western Pomeranian coast, larval traps were installed at regular time intervals, while at the same time water temperature and salinity were measured continuously every hour. Correlations between measured abiotic parameters and borehole abundance of *T. navalis* were tested. For the German Baltic Sea coast, no range expansion of *T. navalis* was confirmed. The salinity and temperatures at the groin systems varied among the study years, and significant correlations between *T. navalis* borehole abundance and salinity as well as temperature were found. Higher summer temperatures favor the *T. navalis* borehole abundance on the Mecklenburg-western Pomeranian coast, and may slightly shift the distribution border of this species toward lower salinities.

Keywords: marine wood-borers, shipworm, Baltic sea, temperature, salinity

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INTRODUCTION

Wooden groins have been an important and common coastal-protection measure in the Mecklenburg-western Pomeranian section of the Baltic Sea coast for 150 years. Traditionally, local pinewood was used until the beginning of the 1990s. At that time, the invasive shipworm *Teredo navalis* Linnaeus 1758 (Bivalvia: Teredinidae) established stable reproductive populations in the southern Baltic Sea, and since then has regularly destroyed the wooden coastal and harbor infrastructure (Sordyl et al., 1998). However, it remains unknown which factors led to the permanent establishment of this shipworm species on the Mecklenburg-western Pomeranian Baltic Sea coast. Periodic mass outbreaks of *T. navalis* in the Baltic Sea were reported previously from Denmark, Sweden, and Germany, during the 1930s and 1950s, each lasting for only a few years (Bavendamm and Schmidt, 1948; Nakel, 1954; Hahn, 1956; Schütz, 1961). However,

since the 1990s pinewood groins have had to be renewed regularly at intervals of 5–10 years, compared to 30–40 years previously (Michael Bugenhagen, StALU-MM: Staatliches Amt für Landwirtschaft und Umwelt Mittleres Mecklenburg—Governmental Agency for Agriculture and Environment Central Mecklenburg-western Pomerania, personal communication). Information on the resulting maintenance costs is sparse; Wichmann (2005) estimated expenses due to *T. navalis* infestations of about 50 million Euros in the Baltic Sea between 1993 and 2005. On the Mecklenburg-western Pomeranian coast, groins are today constructed mainly of tropical hardwoods, which are less attractive to shipworms. Even though the wood used is FSC-certified, the long transport distances may leave a negative ecological footprint. Furthermore, *T. navalis* is threatening wooden shipwrecks and other maritime-related structures of high archeological value (Björdal Gjelstrup et al., 2012). So far, most of these historical items are protected from shipworm attack because they are located in low-salinity waters that cannot be tolerated by this species. Recently, however, contradictory reports have appeared as to whether *T. navalis* is spreading into previously uninhabited areas, even into low-salinity waters (Hoppe, 2002; Tuente et al., 2002; Gollasch et al., 2009; Paalvast and van der Velde, 2011b; Borges, 2014; Appelqvist et al., 2015a).

Shipworms, members of the bivalve family Teredinidae, efficiently consume most kinds of wood in coastal habitats worldwide (Borges, 2014, and references therein). They bore into the wood physically by movements of their reduced shells, and digest the resulting wood particles with the aid of bacterial endosymbionts that produce cellulolytic enzymes and provide fixed nitrogen (Carpenter and Culliney, 1975; Distel et al., 2002). In European coastal waters, at least 9 shipworm species have been reported (Borges, 2014), although species identities are uncertain and molecular genetic characterization is incomplete (Borges et al., 2012; Weigelt et al., 2016). Based on a new COI (cytochrome c oxidase I) primer pair for identification by DNA barcoding, Weigelt et al. (2016) showed that *T. navalis* is presently the only teredinid species on the southwestern Baltic Sea coast. According to Borges et al. (2014) this species is one of the most destructive wood-borers worldwide.

In the Baltic Sea, the earliest known description of *T. navalis* dates to 1835, from Kiel Bay (Meyer and Möbius, 1865), and no scientific evidence exists that places *T. navalis* in the North Sea before 1730. The species, therefore, is classified as invasive in these waters (Leppäkoski and Olenin, 2000; Gollasch and Nehring, 2006). The distribution of *T. navalis* as a marine species depends to a large extent on the prevailing salinity conditions. The minimum salinity required for the survival of adult *T. navalis* is 5–6 (Blum, 1922; Nair and Saraswathy, 1971; Hoagland, 1986), and a maximum of 35 (Nair and Saraswathy, 1971) to 39 (Borges et al., 2014) is tolerated. Individuals show activity at salinities between 7 and 35 (Hoagland and Crocket, 1982; Spicer and Strömberg, 2003; Borges et al., 2014) and successful reproduction is presumed to occur above a salinity of 10 (Schütz, 1961; Soldatova, 1961; Sordyl et al., 1998; Tuente et al., 2002). Larvae survive salinities as low as 5 (Hoagland, 1986). Beside salinity conditions, however, temperature seems to play an important role for the destructive success of shipworms (Kristensen, 1969; Eckelbarger and Reish, 1972). Kristensen (1969), for example, found indications that high temperatures could compensate for low salinities within certain thresholds. The optimum temperature for growth and reproduction is between 15 and 25°C (Roch, 1932; Grave, 1942; Kristensen, 1979). Below 10°C metabolic activity decelerates and stops at temperatures below 5°C (Roch, 1932).

T. navalis is a protandric hermaphrodite species with planktonic veliger larvae. About 5 days after fertilization, the larvae are released into the water and remain there between 15 (Culliney, 1975) and 35 days (Schütz, 1961; Nair and Saraswathy, 1971), probably depending on the availability of wood. After settlement, the larvae metamorphose and bore into the wood quickly. Seven to 8 weeks after metamorphosis, at a length of 3–5 cm, the individuals can be sexually mature (Grave, 1942). Thus, under favorable conditions, more than one reproductive cycle per year is possible. This rapid sexual maturity of *T. navalis* and the very high reproductive potential, with several million larvae per year (Sordyl et al., 1998), allow wide dispersal by currents and efficient discovery of available wooden substrates.

The Baltic Sea is a semi-enclosed sea connected to the North Sea by two narrow and shallow straits, the Belt Sea and the Sound (Mohrholz et al., 2015). In the investigated area of the Baltic Sea a maximum tidal height of about 10 cm is estimated (Medvedev et al., 2016). However, changes of water level fluctuations driven by internal waves and air-pressure changes can be much higher and reach a maximum of 1.5 m annually in the Baltic Sea (Jerling, 1999). The average prevailing surface current along the southern Baltic Sea shoreline flows from west to east (http://www.stalu-mv.de/mm/Themen/Küstenschutz/Regelwerk-Küstenschutz-Mecklenburg-Vorpommern/). This aquatic ecosystem has horizontal and vertical salinity gradients. The salinity drops from 30–25 in the western parts (Belt Sea), to 8–15 in the southern parts (German Baltic Sea coastline) and even to fresh water in the northernmost part (Gulf of Bothnia). Inflows of saline waters from the North Sea occur only sporadically and sustain the brackish character of Baltic waters (Mohrholz et al., 2015). This limited water exchange with high-salinity waters from the Atlantic has a crucial influence on the environmental conditions in the Baltic Sea (Elken and Matthäus, 2008). Mainly, the salinity and oxygen supply affect the benthic community in the brackish-water system of the Baltic (Rönnberg and Bonsdorff, 2004). Due to the decreasing salinity, the number of marine species declines significantly in the southern Baltic and in the inner coastal waters (Zettler and Röhner, 2004; Bonsdorff, 2006). In the Mecklenburg-western Pomeranian part of the Baltic Sea coast, there is a horizontal salinity gradient between about 12 in the westernmost part and 7–8 around the island of Rügen. Since 1993, *T. navalis* has been permanently present in this area (Sordyl et al., 1998). For a long time it was assumed that the Darß Sill, with a salinity of around 10, was the easternmost distribution border for *T. navalis* in the Baltic Sea. In 1998, however, Sordyl et al. (1998) provided evidence for permanent *T. navalis* infestations east of the Darß Sill, at Zingst and on the western coast of the island of Hiddensee.

They, however, did not detect *T. navalis* infestations farther east on the islands of Rügen and Usedom. Furthermore, Sordyl et al. (1998) found that the population was successfully reproducing, which had not been expected before due to the prevailing low salinities.

The mean Baltic Sea surface temperature from July to September has increased by 1.4°C between 1985 and 2000 (MacKenzie and Schiedeck, 2007). As mentioned above, the survival and distribution of *T. navalis* is mostly affected by salinity-temperature interactions (Kristensen, 1969; Borges, 2014). The rise in water temperature since 2000 has further increased by 0.6°C per decade; this trend will continue (Siegel and Gerth, 2016), and might thereby shift the eastern distribution border of this species farther. Since 1998, however, no investigation of the distribution of *T. navalis* on the Mecklenburg-western Pomeranian coast has been carried out and information on a potential range expansion, for example, to the island of Rügen, is lacking.

The particular abiotic conditions in the Baltic Sea, with a salinity gradient from west to east, provide a good opportunity to study *T. navalis* at its distribution border. Accordingly, for the first time in 17 years we systematically surveyed the occurrence of *T. navalis* in groin systems on the Mecklenburg-western Pomeranian coast over a period of 4 years, from 2012 to 2015. Temperature and salinity conditions in the shallow water areas investigated were simultaneously recorded, with high temporal resolution, to evaluate a potential expansion of the salinity and temperature range required by *T. navalis*. We also studied the start and duration of spat fall in relation to the spring temperature increase and summer temperatures. In 2014, a major intrusion of saline water from the North Sea to the Baltic occurred (Mohrholz et al., 2015). This provided an opportunity to investigate if these sporadic events improve the salinity conditions for *T. navalis* in the Mecklenburg-western Pomeranian part of the Baltic Sea.

MATERIALS AND METHODS

Study Area and Larval Sampling

Along the Mecklenburg-western Pomeranian coastline of the Baltic Sea, seven locations were sampled between 2012 and 2015, from Boltenhagen (N53°59.877′, E11°11.722′) to the west and Glowe (N54°34.219′, E13°27.571′) on the island of Rügen to the east (**Figure 1**). Locations were chosen to assess the abundance of *T. navalis* boreholes and the time of larval settlement along the salinity gradient prevailing along this section of the Baltic Sea coast. The sampling sites mainly have a soft sandy bottom, partly interspersed with gravel and stones.

Panels of unplaned pine wood (*Pinus sylvestris*, 10 × 20 × 2.5 cm), suitable as larval traps for teredinids (Borges et al., 2014), were attached to wooden pilings of the groin system by scuba divers, using cable ties. The panels were provided with a hole in each corner, at about 2.5 cm distance from the edges of the panel. Two cable ties (0.9 × 150 cm) (**Figure 2**) were drawn from the back side of the panel through one hole each, laid in parallel over the front surface of the panel, drawn through the opposite hole, and fixed to a groin piling. The cable ties covered 25 cm^2 of the total panel surface of 200 cm^2. Depending on the sampling site, the panels were placed at a depth between 1 and 3 m, about 0.5 m above the sea floor and distributed on three different rows of pilings in the respective groin system, with a distance of at least 50–70 m between the panels.

The total number of boreholes during one summer was quantified by exposing three larval traps per site each year from May to September or October. In total 84 panels were exposed for this purpose. To determine the time of larval settlement on the wood, three additional panels per site were exposed and replaced monthly between May and September or October, respectively. Between October and May, three panels per site were exposed to check for larvae arriving later in the year than

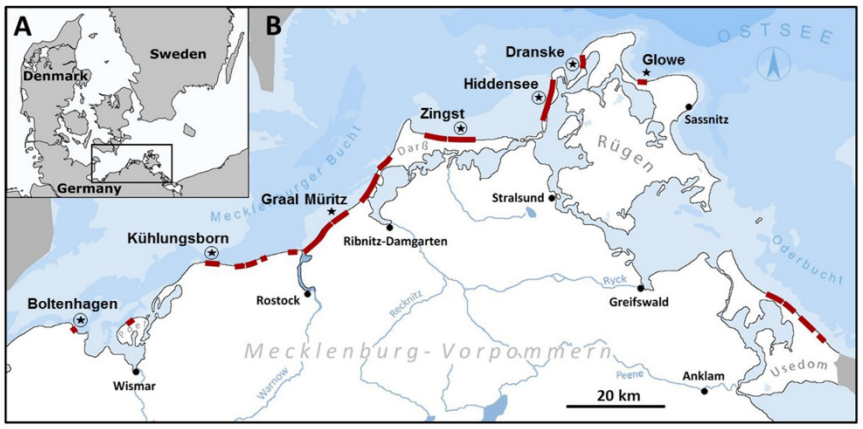

FIGURE 1 | Sampling area: **(A)** Position of the sampling area on the southern Baltic Sea coast. **(B)** Sampling area: Red bars indicate groin systems in the sampling area. Stars mark sampling locations with only test panels, and stars with circles indicate sampling locations where test panels and data loggers were installed in combination.

October. This means that 504 panels were exposed and recovered to determine the time of larval settlement, giving a total number of 588 examined panels in the present investigation. After the defined exposure times, panels were recovered by scuba divers, gently cleaned of fouling organisms, blotted dry with tissue paper and air-dried for about 1 h. Next, the total number of boreholes on the panel front side, oriented toward the open sea water (~200 cm^2), was counted using a stereomicroscope. On the panel front, sections were marked with a pencil. Each section was examined for boreholes, using a magnification of 6.7–10×. A typical borehole had a diameter of 400–700 µm. In 2013 and 2014, the density of boreholes at several sites was extremely high, making it impossible to count each hole separately. In these cases, the panel front was divided by a raster of 2.25 cm^2. From this raster, 15 squares were randomly chosen and the boreholes in it were counted. The resulting number was extrapolated to the total surface of the sea water-exposed panel front.

According to Weigelt et al. (2016), *T. navalis* is the only teredinid species on the Mecklenburg-western Pomeranian Baltic Sea coast. In the present study, species identification of voucher specimens from all sampling locations was conducted by DNA barcoding as described by Weigelt et al. (2016). *T. navalis* was confirmed to be the only shipworm species in this area.

Abiotic Parameters

Salinity and temperature were measured hourly at three sites in 2012 (Boltenhagen, Zingst, Dranske) (**Figure 1**), at four sites in 2013 and 2015 (Boltenhagen, Kühlungsborn, Zingst, Dranske) and at five sites in 2014 (Hiddensee additionally). Data were obtained by autonomous Conductivity-Temperature Data Loggers (HOBO—U24-002, Onset Ltd., Cape Cod, Massachusetts, USA), attached to the groins by cable ties next to the wooden panels, about 0.5 m above the sea floor. According to the manufacturer, the specific-conductance accuracy for salinity measurements is about 3%, and that for temperature measurements ± 0.1°C. Although the data loggers were cleaned of fouling organisms every 4 weeks during the summer months, recolonization of the salinity sensor sometimes occurred rapidly and led to partial loss of data. All results were examined visually for consistency, and conspicuous data points that clearly indicated the influence of fouling were not used for further analysis. Temperature measurements were not affected by fouling.

Statistical Analysis

Abiotic data for the statistical analysis were collected at the Boltenhagen, Kühlungsborn, Zingst, Hiddensee, and Dranske stations. Borehole abundance data include all stations investigated. All statistical analyses were carried out with the statistical software R version 3.3.0 (R Development Core Team, 2009). Daily mean water temperature and salinity were calculated from the hourly data. To reveal significant differences in the parameters summer temperature (21 June to 20 September), salinity and *T. navalis* borehole abundance among sampling stations and years, a one-way Anova followed by the *post-hoc* Tukey HSD test was applied.

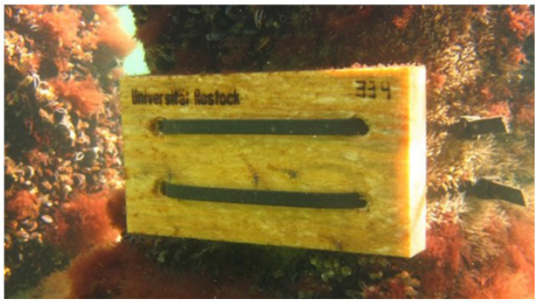

FIGURE 2 | Larval trap: Representative pinewood panel attached to a groin in the shallow water of the southern Baltic Sea coast.

Significant correlations between borehole abundance at different sites and in different years and the environmental parameters water temperature and salinity were assessed by the Spearman's rank correlation coefficient. To rate the significance of the correlation, the *p*-value of the *F*-statistics was used. The following parameters were tested for significant correlation with borehole abundance: mean annual salinity, mean summer salinity and mean summer temperature, number of days with mean salinity below 8, number of days with mean salinity above 10 and above 12, number of days with mean temperature above 18°C, above 20°C and above 22°C. These parameters were chosen to reveal possible responses of *T. navalis* to certain salinity thresholds and to summer temperature peaks. Salinity of 8 is close to the assumed lower activity threshold of *T. navalis* (Hoagland and Crocket, 1982; Spicer and Strömberg, 2003), 10 and 12 are slightly above. Daily mean water temperatures above 20°C or even 22°C are not reached very often in the Baltic Sea during the course of the study, however higher temperature may favor the success of *T. navalis* in low salinity waters (Kristensen, 1969; Eckelbarger and Reish, 1972).

RESULTS

Salinity and Temperature

Salinity conditions and water temperatures measured during the 4 years of investigation at different sites of the Mecklenburg-Western Pomeranian coast are shown in **Figures 3**, **4**, and in **Table 1**. The stations follow the salinity gradient in the Baltic Sea and are significantly different in their mean salinity over the 4 years (ANOVA, $p < 0.0005$). The mean salinity over 4 years is plotted exemplarily for the stations Boltenhagen in the west and Dranske in the east of the sampling area (**Figure 3**). While at Boltenhagen, the salinity was mostly above 10 and reached values up to 17 during the winter months, at Dranske salinity ranged between 8 and 10 most of the time, but also periods with salinity below 8 were registered. The annual mean values for salinity (**Table 1**) showed highest values at Boltenhagen and Kühlungsborn with a mean yearly salinity between 12 and 14 and about 90% of the data points above a salinity of 10. Farther east, at Zingst, the mean summer salinity mainly ranged between 9

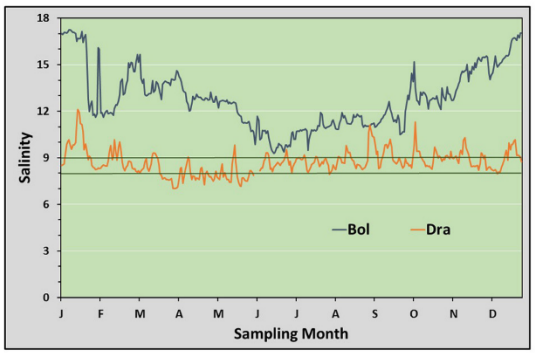

FIGURE 3 | Salinity: Mean salinities based on data collected from 2012 to 2015. The westernmost (Boltenhagen) and the easternmost (Dranske) sampling sites are shown. The limiting lower salinity for *Teredo navalis* (between 8 and 9) is indicated by dark-green lines.

and 10. More than 30% of the daily mean salinities measured at Zingst during summer showed values above 10 in all of the years. Nevertheless, the number of days above a salinity of 10 is variable between years and even days with a mean salinity below 8 were observed (11%). At Dranske the frequency of mean daily salinities below 8 (5–11% of measured summer days) was similar to those at Zingst. However, the percentage of days with measured salinities above 10 was lower than at the more western location Zingst and the annual mean salinity at Dranske varied between 8 and 9. At the sampling stations Boltenhagen, Kühlungsborn, and Zingst the salinity did not change with the saltwater intrusion in 2014 (**Table 1**). During the investigated 4 years no relevant daily fluctuations in salinity were observed.

During all of the investigated years temperatures above 15°C were reached between late May and early June and dropped below this value between mid of September and mid of October. The most striking differences between the years studied were the low water temperatures in winter 2012/13 lasting until mid of April 2013 and the long lasting high water temperatures in summer 2014 (**Figure 4**). The mean water temperature between 21th of December and 20th of March in 2012/13 was 2.7°C compared to 5.5°C in 2013/14 and 5.8°C in 2014/15. Despite this low mean water temperature, there were only few days between December 2012 and April 2013 with an mean daily temperature below 0°C (Boltenhagen 1; Zingst 14). During winter 2013/14 at 2 days mean temperatures below 0°C were measured, while in 2014/15 temperatures below 0°C were not reached at all.

The summer mean temperatures at Boltenhagen were highest in all years (19.3°C, four-year mean), while at Dranske they were always lowest (17.6°C, 4-year mean) (**Table 1**). Also during summer months, water temperatures at Dranske dropped below 15°C for short intervals (**Figure 4**), which might be due to upwelling events of colder bottom water in this region. At Boltenhagen the highest maximum values of summer water temperatures were measured, often reaching daily mean values of 22°C (**Table 1**). The summer mean temperatures including all stations differed significantly from each other, except Kühlungsborn and Zingst (ANOVA, $p < 0.0005$). In 2014, mean water temperatures were the highest at all stations. Furthermore, water temperatures above 15°C continued until October 20, which was 4 weeks longer compared to 2012 and 2013 and 2 weeks longer than in 2015. Also the summer means in the 4 years differed from each other: 2012 and 2015 were significantly colder than 2013 and 2014 (ANOVA, $p < 0.0005$).

Borehole Abundance

T. navalis boreholes in submerged pine wood panels were observed at all stations between Boltenhagen and Zingst during each of the 4 years (**Figure 5**). Farther east, at Hiddensee, Dranske, and Glowe, boreholes were found less frequently or were absent. At Glowe, no larval boring activity was observed on summer-exposed panels. However, in panels exposed at Glowe from late September or October to spring of the following year, several boreholes were found, indicating a late arrival of larvae (**Figure 6**). At both of the easternmost sampling sites, Dranske and Glowe, boreholes were always empty or with dead larvae, indicating unsuccessful recruitment. The borehole abundance was significantly lower in Dranske and Glowe compared to the other stations (ANOVA, $p < 0.05$).

The number of boreholes of *T. navalis* on the surface of the exposed pine wood varied considerably among locations and years, with the highest number of boreholes counted in 2014 (Median: 357 boreholes dm^{-2} between Boltenhagen and Zingst) and the lowest in 2015 (Median: 40 boreholes dm^{-2} between Boltenhagen and Zingst). The borehole abundance in 2015 differed significantly from the abundance in 2014 (ANOVA, $p < 0.05$). The highest abundance was found at Kühlungsborn and Graal Müritz during summer 2012 und 2013, while at Boltenhagen and Zingst it was comparatively low in these years. This pattern changed in 2014, showing a maximum at Boltenhagen in the west, and lower numbers at Kühlungsborn, Graal Müritz, and Zingst (**Figure 5**).

Influence of Temperature and Salinity on *T. navalis* Borehole Abundance

Significant correlations between the borehole abundance of *T. navalis* and seawater temperature or salinity revealed by Spearman's rank correlation test are shown in **Table 2**. The tests including the data from the Boltenhagen, Kühlungsborn, Zingst, and Dranske stations over all study years indicate significant correlations between borehole abundance and summer mean temperatures, as well as between borehole abundance and summer mean salinity. Salinity and temperature are not co-correlated in our data set. On the other hand, in the tests concerning data from single stations, significant correlations between borehole abundance and temperature were found only for Boltenhagen and Zingst. No correlations between borehole abundance and salinity were found by the single-station tests. Since a combined effect of temperature and salinity on *T. navalis* distribution seems probable in the Baltic Sea, an ANOVA was also applied, but did not yield any significant information on the variance.

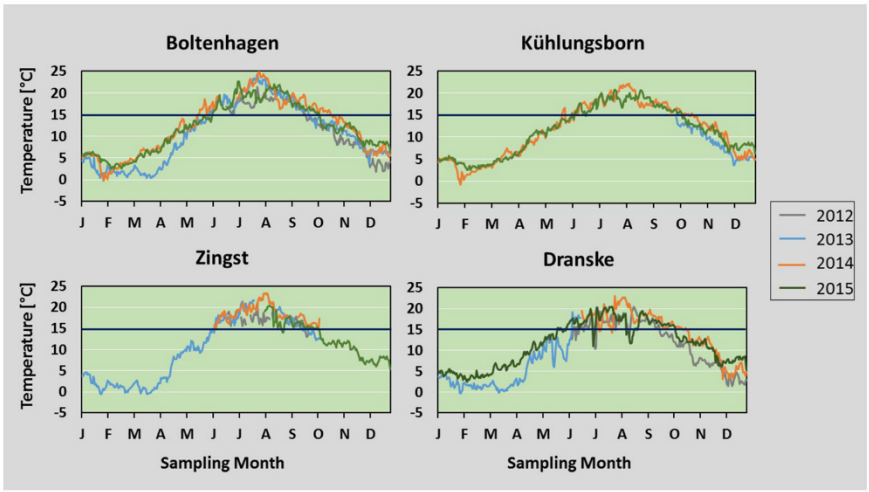

FIGURE 4 | Temperature: Daily mean temperatures measured from 2012 to 2015 at Boltenhagen, Kühlungsborn, Zingst, and Dranske. Values are based on hourly measurement intervals. The dark-blue line indicates the presumed lower threshold of 15°C for the start of *Teredo navalis* reproduction.

Time of Larval Settlement

From the monthly sampling of larval traps, a peak period of settlement per year and sampling site was defined (**Figure 6**). The main settling period was considered as the time when the number of boreholes was higher than 10% of the total boreholes of the respective year and sampling location, and it was distinguished from the periods when only single larvae attached to the wood. This period of intensive wood attack by *T. navalis* differed only slightly among the sampling locations, but differed more among years. The earliest boreholes were found on the panels by the end of June (2014). The main larval settlement started in the middle to end of August (2012, 2013), and in mid-July (2015) to the beginning of August (2014, 2015). The main settlement period in 2013 and 2014 was relatively short compared to 2012 and 2015. In 2013 it lasted until mid-September, over a total period of 2–4 weeks, depending on the site (**Figure 6**). In 2015 newly settled larvae were found on the panels until the beginning of September, a period of 4–5 weeks. At Kühlungsborn, larvae were settling even over a period of 9 weeks. During 2012 and 2015, an intensive *T. navalis* attack continued until October. Individual larvae were found on the panels until the end of October in both years.

At Glowe, no larvae settled on the exposed panels during summer of the study years. In late September 2013 and late October 2014, however, larvae bored into the panels at Glowe, but did not survive. Similar observations that larvae could not settle successfully after September were made at the other locations investigated.

The main settling period of *T. navalis* larvae began 5–12 weeks after the water reached a mean weekly temperature above 15°C (**Figure 6**), a threshold for larvae release into the water in other regions (Loosanoff and Davis, 1963; Culliney, 1975).

DISCUSSION

For several years, the possibility of a range expansion by *T. navalis* has been debated in the literature. Some authors have provided evidence that this species is or will be moving into formerly uninhabited areas, due to changes in hydrographic conditions caused partly by climate change (Hoppe, 2002; Tuente et al., 2002; Gollasch et al., 2009; Paalvast and van der Velde, 2011b; Borges, 2014). Other authors found that this is not the case for certain geographic areas such as the western coast of Sweden (Appelqvist et al., 2015a,b). For the southwestern Baltic Sea, Borges et al. (2014) postulated a range expansion of *T. navalis* by adaptation to salinities as low as 7. Adaptation of this species to lower salinities, however, was not confirmed experimentally. The present distribution border of *T. navalis* for the southwestern coast of the Baltic Sea is, according to the results of the present study, located east of the island of Hiddensee at salinities between 8 and 9. This coincides with the findings of Sordyl et al. (1998), implying that no range expansion has taken place during the last 17 years. However, from our results it is reasonable to assume that the actual distribution border is not strict, since slight changes in salinity or temperature may allow *T. navalis* to settle and reproduce farther east.

T. navalis was regularly found in high numbers between Boltenhagen in the west and Zingst in the east on the Mecklenburg-western Pomeranian coast (**Figure 1**). At Zingst, the annual mean salinity ranges between 9 and 10.8 (**Table 1**). Farther east at the island of Rügen, only sporadically low numbers of boreholes were found, even though the annual mean salinity at Dranske was only slightly lower in 2014 and 2015 compared to Zingst. The four-year mean salinity was higher at Zingst (9.7) than at Dranske (8.8). For the survival and growth of *T. navalis*, however, not the mean yearly salinities *per se* might be important,

TABLE 1 | Abiotic variables at the different sampling locations in the years 2012–2015.

		2012				2013				2014				2015				2012–15			
		Bol	Küh	Zin	Dra	Bol	Küh	Zin	Dra	Bol	Küh	Zin	Dra	Bol	Küh	Zin	Dra	Bol	Küh	Zin	Dra
Annual mean salinity		12.6	n.d.	10.8	8.7	11.9	n.d.	9.2	8.1	13.5	13.9	8.9	9.1	13.0	12.5	9.8	9.5	12.8	13.5	9.7	8.8
% days mean salinity																					
	<8	0	n.d.	0	6	0	n.d.	12	59.0	0	0	22	10	0	0	12	0	0	0	11	18
	>10	99	n.d.	69	3	67	n.d.	29	3.0	99	96	19	19	91	89	64	25	90	93	43	12
	>12	54	n.d.	19	0	42	n.d.	4	0.0	73	73	0	4	56	44	0	5	58	59	7	2
Number of days included		189	0	72	187	224	0	75	153	342	285	63	188	356	271	33	126	1111	556	243	654
Mean summer temperature		18.0	n.d.	17.4	16.9	19.7	n. d.	18.1	n. d.	20.3	18.8	19.6	18.7	19.3	18.3	17.1	17.4	19.3	18.6	18.2	17.6
% days mean temperature																					
	>18°C	53	n.d.	19	21	69	n.d.	34	n.d.	71	50	70	61	67	57	11	39	260	107	143	121
	>20°C	5	n.d.	2	1	42	n.d.	13	n.d.	40	25	33	20	41	9	6	10	36	18	20	11
	>22°C	0	n.d.	0	0	19	n.d.	0	n.d.	22	2	14	7	2	0	0	0	12	1	5	3
Number of days included		92	0	65	92	92	0	72	0	80	92	91	92	92	92	47	92	356	184	275	276

Bol, Bolterhagen; Küh, Kühlungsborn; Zin, Zingst; Dra, Dranske; n.d., not determined.

but rather the length of the time intervals above a certain salinity threshold. At Zingst, for example, the percentage of summer days with mean salinities above 10 was higher (43%) compared to Dranske (12%) in all years (**Table 1**). At Hiddensee the salinities were above 10 on 31% of the days measured, although only a few data points were collected from this station.

As mentioned above, Borges et al. (2014) found *T. navalis* settling on exposed wooden panels at a salinity of 7 in the Mecklenburg-western Pomeranian part of the Baltic Sea. The site described by Borges et al. (2014) (N 54°24.599′, E 12°36.6′), however, is located in the inner coastal waters of the Baltic Sea west of Zingst, in the Bodstedter Bodden which is part of the Darss-Zingst-Bodden-Chain (Schiewer et al., 1994). The salinity at the sampling location given by Borges et al. (2014) is based on information from a global hybrid data set, compiled primarily from the environmental dataset in BIO-ORACLE (www.oracle.ugent.be/). These values were not confirmed by daily long-term measurements (1979–2004) in the Bodstedter Bodden provided by Schumann et al. (2006), which show a median salinity of 5.4. Borges et al. (2014) described the sampling location as a wreck site in the mouth of the river Prerowstrom. In view of the low salinity in the Bodstedter Bodden and the absence of wrecks in this area, we find it highly unlikely that *T. navalis* was found in this part of the inner coastal waters of the Baltic. We suppose that the panels of Borges et al. (2014) were exposed ca. 2 km north of the Bodstedter Bodden at the former mouth of the Prerowstrom, which entered the open Baltic Sea until it was closed by sediments after a strong storm in 1872. At this location, several wrecks are located (among others the Darß Kogge), and prevailing salinities are similar to those measured at Zingst, making the presence of *T. navalis* highly likely there.

Dranske and Glowe, located on the island of Rügen, are the easternmost sampling sites and therefore those with the lowest salinity investigated in the present study. During the regular sampling described above, several boreholes were found at Dranske in three of the 4 years, even though the abundance was comparatively low, with one to four boreholes per test panel ($\leq$1 dm^{-2}). At Glowe, farther east than Dranske (**Figure 1**), boreholes were detected only in 2012 and 2013, with abundance levels similar to that at Dranske. Even though boreholes were present and salinities were mainly above 8, at least at Dranske, no living larvae or adult specimens were ever observed. During a *T. navalis* survey conducted in 2016, however, adult shipworms were unexpectedly found in older wooden panels exposed in 2013 and 2014. Specimens were up to 20 cm long (shipworm length, not tube length) and an abundance of three to five individuals per panel (about 2 dm^{-2}) could be documented (own results, not shown here). According to the size of the individuals, they probably had already settled in 2013 or 2014, due to slow growth in low-salinity waters. Considering the salinity conditions, Dranske is located at the distribution border of *T. navalis* in the southwestern part of the Baltic Sea. Thus, even slight changes in the abiotic conditions can make this particular habitat favorable or unfavorable for the wood-borer.

The mean annual salinity at Dranske varied between 8.1 in 2013 and 9.5 in 2015, showing no clear relationship to borehole abundance in the respective year. The mean temperature in

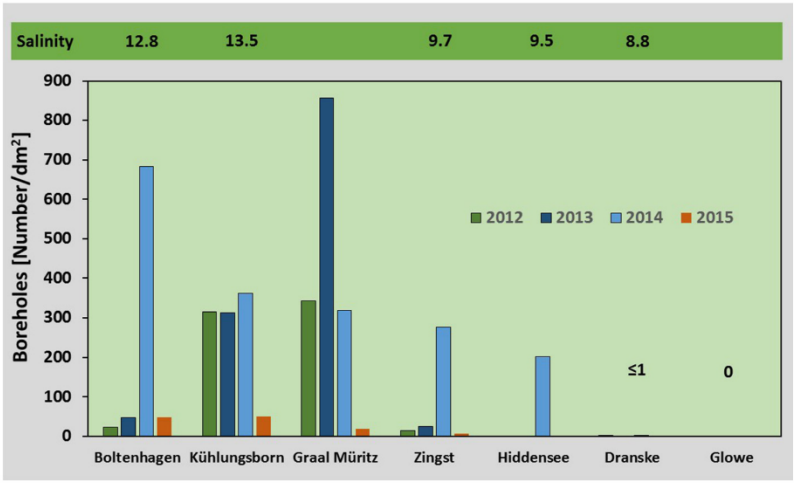

FIGURE 5 | Larvae abundance: Bars indicate the median ($n = 3$) number of boreholes counted per dm^2 on test panels at the different sampling sites in the years 2012–2015. Panels were exposed from May to October. The mean salinity based on measurements from 2012 to 2015 (hourly data) is given above the bars.

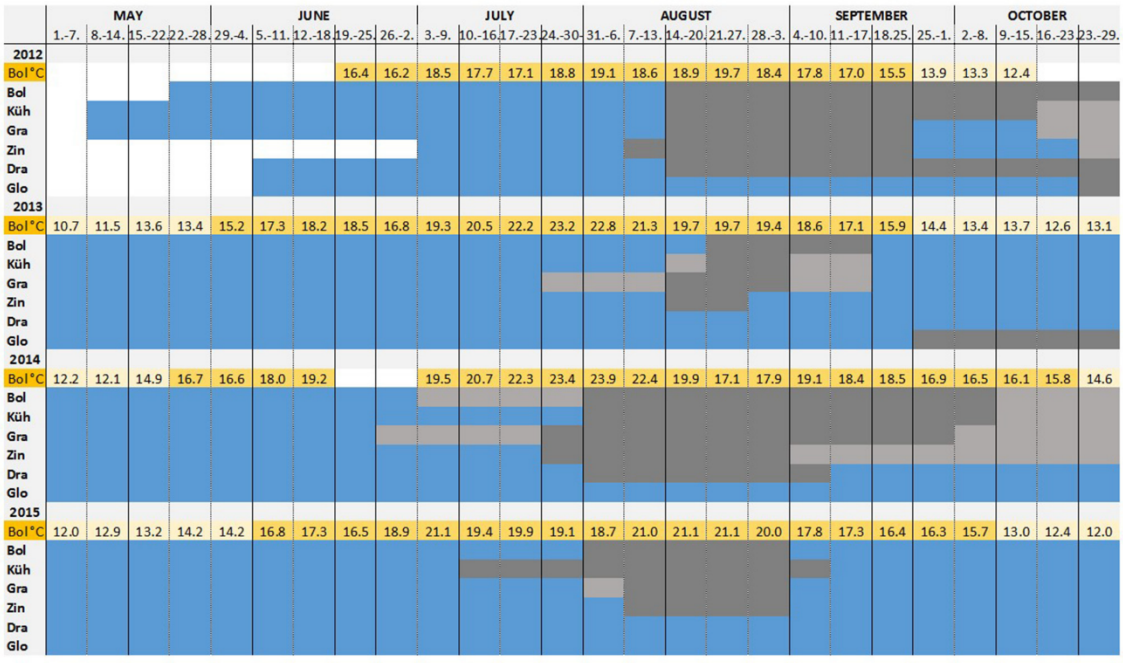

FIGURE 6 | Time of larval settlement: During each month in summer, a test panel was collected and replaced by a new one. Periods of high, low and no *Teredo navalis* recruitment are shown. The mean temperature per week measured at Boltenhagen is indicated above the bars, showing the temperature variation during the summers of 2012–2015. Temperatures above 15°C are highlighted. The period of low recruitment is defined as the period when less than 10 percent of total larvae of the respective year and sampling location settled. no recruitment main recruitment period low recruitment period no data.

TABLE 2 | Spearman's rank correlation coefficient for potential influence of seawater temperature and salinity on the abundance of *Teredo navalis* on the Mecklenburg-western Pomeranian coast.

	Larval abundance vs.	Spearman's rank correlation coefficient
All years, all stations	**Mean summer temperature**	0.72***
	Mean summer salinity	0.79***
Boltenhagen	**Mean summer temperature**	0.77**
Zingst	**Mean summer temperature**	0.88*

*Significance level: *<0.05, **<0.01, ***<0.001. Coefficients above a threshold of 0.7 reflect correlations between shipworm abundance and the respective abiotic factor, and are given in bold.*

summer 2014, however, was 1.8 and 1.3°C, respectively, higher than in 2012 and 2015. The warm summer temperatures in 2014 lasted until October 21, thus 2–4 weeks longer than during the other years investigated. During the summers of 2012, 2013, and 2015, water temperatures at Dranske abruptly dropped below 15°C from time to time. Such rapid decreases were not observed in summer 2014 (**Figure 4**). Since reproduction, survival, and metamorphosis of *T. navalis* larvae are temperature-dependent (Nair and Saraswathy, 1971; Hoagland, 1986), we suggest that the higher and more stable temperatures in 2014 favored the establishment of *T. navalis* larvae and the growth of juveniles, although in terms of salinity, this species is at its lower physiological limit. For other teredinids (*Lyrodus pedicellatus* Quatrefages 1849) the importance of combined temperature-salinity effects was asserted by Eckelbarger and Reish (1972). Kristensen (1969), working in Danish waters, found indications that *T. navalis* attacks were most likely to take place when summer mean temperatures were above 19°C, and such attacks were strongest when high water temperatures coincided with increased salinities. Applying a climate-envelope model, Appelqvist et al. (2015a) found for the western Baltic Sea that favorable conditions for *T. navalis* were controlled by temperature whenever salinity was above 8. This result is supported by the present study through field investigations of *T. navalis* abundance combined with direct measurements of temperature and salinity conditions at the sampling locations. However, from the present data, this effect cannot be asserted statistically. The decreasing salinity from west to east along the Mecklenburg-western Pomeranian coast is confounded with decreasing temperature, making the chosen sampling spots inappropriate to test this hypothesis. Further investigation at selected sampling locations as well as laboratory experiments under controlled temperature and salinity conditions would be necessary.

Optimum temperatures for reproduction of *T. navalis* are above 15°C (Roch, 1932; Kristensen, 1979). Larvae are released at the straight-hinge veliger stage, about 5 days after fertilization (Culliney, 1975) and remain in the water column for another 15–35 days before settlement (Schütz, 1961; Nair and Saraswathy, 1971; Culliney, 1975). On the Mecklenburg-western Pomeranian coast, temperatures above 15°C were reached in early June in all of the years investigated. According to these observations and to the literature cited above, larvae would have been expected to start settling on the exposed panels between late June and early July. This was not the case in the present study, with few exceptions (see **Figure 6**). The settling period started 5–12 weeks after the optimum temperature of 15°C was reached. There was thus no obvious relationship between the threshold temperature persumed to trigger larval release and the settling period of *T. navalis*. Similarly, Paalvast and van der Velde (2011a) found *T. navalis* breeding from August to September in the Port of Rotterdam area, even though favorable conditions for reproduction were reached in April or May. Furthermore, Kristensen (1979) found *T. navalis* larvae settling in the Danish Isefjord from mid-August to the end of September. In contrast, on the Swedish west coast, larvae settled from the end of June to the beginning of July (Appelqvist and Havenhand, 2016), thus mainly several weeks earlier than on the Mecklenburg-western Pomeranian coast. Appelqvist and Havenhand (2016) established a significant correlation between the date at which the mean sea surface temperature reached 16°C and the start date of intensive recruitment. The results from the present study, as well as from Kristensen (1979) and Paalvast and van der Velde (2011a) may indicate that this temperature threshold is not relevant for all regions, or that abiotic factors other than temperature influence the start of larval settlement. These factors might be lower salinities, for example, compared to the Swedish west coast, or the availability of food or wood. Furthermore, prevailing wind direction and intensity influence coastal currents and may determine the settling success of released larvae. These influences, as well as the prevailing period of *T. navalis* larvae in the coastal waters of the southwestern Baltic Sea, were not analyzed in the present study. Therefore, no clear trend was found for the settling time of *T. navalis* larvae on the Mecklenburg-western Pomeranian coast during 2012 and 2015. However, different authors (Ryabchikov and Nikolaeva, 1963; Kristensen, 1979) did find that peak settling coincided with the end of a period of high temperatures. This relationship could not be confirmed by the present investigation, since sampling intervals of 4 weeks were too long for this purpose. Nevertheless, a tendency to intensive settlement toward the end of a period of warm temperatures can be hypothesized from the results, and should be investigated in more detail in future studies.

Compared to investigations on *T. navalis* from other coastal regions, the borehole abundance on the panels exposed at the Mecklenburg-western Pomeranian coast seems very high. Maximum numbers of boreholes in the study years were between 990 (2013) and 90 (2015) boreholes per dm^2. Kristensen (1979) found a maximum of 27 juvenile *T. navalis* per dm^2 in Danish waters, and Norman (1977a,b) and Appelqvist et al. (2015b) between 100 and 200 individuals per dm^2, respectively, on the Swedish west coast. However, in these studies juvenile individuals were counted using x-ray radiography, in contrast to the present study, in which boreholes were counted. The latter method leads to an overestimation of larval abundance, since not every borehole was inhabited by a successfully metamorphosed living shipworm. Control counts comparing the number of living individuals with the number of boreholes showed that about 70% of the larvae arriving at a wooden panel successfully settled (own results, not shown here). Even taking this into account, the

number of boreholes in the southwestern Baltic Sea is generally high compared to other regions, also in years with low borehole abundance at the study sites. Also, Zettler et al. (2000) observed that benthic marine species occur in higher abundances in the Mecklenburg Bight, close to their distribution border, than in more marine regions, although the individuals remain smaller due to low salinity. This might be due to a potentially large catchment area for larvae in this part of the Baltic Sea coast, including the German, Danish, and Swedish coasts (Appelqvist et al., 2015a), or to reduced competition for space due to lower biodiversity in low-salinity waters.

The sporadic inflows of high-salinity waters from the North Sea to the Baltic Sea strongly influence the environmental conditions in the Baltic (Elken and Matthäus, 2008), as mentioned in the introduction. One of the major intrusion events of the last century occurred in 1993 (Dahlin et al., 1993; Mohrholz et al., 2015), and coincided with the return and permanent establishment of *T. navalis* along the southwestern Baltic coast (Sordyl et al., 1998). This led to the assumption of several authors, that the salinity increase in the Baltic positively influenced the establishment of *T. navalis* (Sordyl et al., 1998; Nehring, 2005). During the present study the first major saltwater intrusion since 2003 (Mohrholz et al., 2015) was observed. We recorded a sharp, although temporary rise of monthly mean salinities in January 2014 at the Boltenhagen, Kühlungsborn, and Dranske stations. At Boltenhagen and Kühlungsborn the salinity was 18.5 and hence more than 3 units higher than the highest mean value for January measured during the 4 years of the present investigation. At Dranske the rise was more than 2 units up to a salinity of 10.4 (own results, not shown here). In February, salinity returned to levels frequently measured during the respective time of the year. These findings coincide with those of Schumann et al. (2006), who also found that a salinity increase related to salt-water intrusion lasted for only a few weeks, mainly January and February. In 2015 after the major salt-water intrusion, total larval densities were much lower than in other years. At Dranske und Glowe no larvae at all were found in 2015. The major salt-water intrusion in 1993 also occurred during winter, minimizing possible effects on the introduction of *T. navalis* to the Baltic Sea. We therefore conclude that the very sporadic major salt-water intrusions from the North Sea have little or no effect on *T. navalis* in the Baltic Sea, particularly during winter when temperature is too low for the physiological requirements of this wood-borer.

To prevent further spreading of the invasive *T. navalis* along the coastline, Appelqvist et al. (2015a) suggested to remove wooden structures as potential stepping stones. On the Mecklenburg-western Pomeranian coast, the groin systems deployed at short intervals along the shore indeed represent ideal man-made stepping stones for the distribution of *T. navalis* from higher-salinity waters toward the lower-salinity waters farther east. However, it must be noted that the use of wood as a natural building material in the Baltic Sea should be favored for coastal protection and other maritime purposes, and hence is highly recommended by HELCOM (Rec 16-3 Helcom, http://helcom.fi/helcom-at-work/recommendations).

The present investigation did not provide definitive results regarding the factors that might control the settlement of *T. navalis* larvae along the Mecklenburg-western Pomeranian coast. This is partly due to the high variances in borehole abundance. At Boltenhagen, for example, small numbers of boreholes, compared to Kühlungsborn and Graal Müritz, were found in the years 2012, 2013, and 2015, although Boltenhagen should have more favorable conditions for *T. navalis* larvae in terms of salinity and temperature. In 2014, the borehole abundance was much higher at Boltenhagen than in Kühlungsborn and Graal Müritz. In 2014, the number of settling larvae was increased at Zingst and Hiddensee compared to the other study years. At Zingst and Hiddensee the increased borehole density is probably due to the high summer temperature in 2014. The variability at Boltenhagen, however, cannot fully be explained by the abiotic parameters measured. Rather, variability in surface currents, perhaps due to prevailing wind directions in the respective year, may have caused the high larval abundance in 2014. Detailed information on water currents along the Mecklenburg-western Pomeranian coast in the study years would be essential but were not measured in this study. Furthermore, the varying salinity and temperature conditions and the parallel decrease of salinity and temperature toward the east make interpretations difficult. However, the present study was conducted as a first step toward an understanding of the species' occurrence at the limit of its distribution. For detailed analyses of combined salinity-temperature effects, experiments under controlled conditions (laboratory or mesocosm) would be preferable, where salinity and temperature combinations should be chosen according to the results of the present study.

AUTHOR CONTRIBUTIONS

HL has to a great extend performed the field and laboratory work, has analyzed the data and written the first draft of the manuscript. RW has taken part in the field and the laboratory work, has contributed to figure drafts and has edited the manuscript. RK has essentially contributed to field and laboratory work. RB has been involved in project development and has contributed to the manuscript. UK has significantly been involved in project development and realization and has essentially edited the manuscript. All authors have approved the final manuscript.

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4 Discussion

It has generally been ascertained that the infestation of wood in the Baltic Sea with shipworms appears to be subject to a certain spatio-temporal limitation (Cordshagen, 1964). There had been periods of severe attacks followed by periods with little or no infestations which may have lasted for several years or even decades. These strong infestation periods of 2 - 3 years seem roughly to correspond to the life span of *T. navalis* in the waters of Central Europe. In January 1993, a large salt water inflow into the Baltic Sea [Major Baltic Inflow (MBI); Matthäus, 1993] seemed to coincide with the reoccurrence of shipworms in the Baltic Proper (Appelqvist, 2015). In contrast to the previous short-term outbreaks, the permanent occurrence of *T. navalis* since 1993 is an unprecedented state in the southern Baltic Sea.
Several reasons as to why the population has become permanent since the last outbreak can be listed. First, established populations had not existed before, since they had usually collapsed within a certain timespan after the species introduction due to the prevailing living conditions. Secondly, regular monitoring programs did not exist during the 19th and 20th centuries so that even smaller refugial populations, if any existed, remained undiscovered. Thirdly, it is conceivable that an existing population has adapted better to the prevailing environmental conditions or the environmental conditions changed and therefore favored the establishment of a permanent population. Finally, it cannot be ruled out that a new species has migrated to the Baltic Sea showing better coping mechanisms to the prevailing environmental conditions, which resulted in an explosive distribution.

4.1 Species identification and phylogenetic analysis of *Teredo navalis*

Difficulties of shipworm identification

One of the essential issues in working with marine shipworms is an unambiguous, reliable identification and taxonomic classification of the different teredinid species. This is mainly due to two factors: their hidden way of life in calcareous living tubes and the lack of appropriate trustworthy, classical morphological taxonomic features, other than the pallets (Turner, 1966; Borges *et al.*, 2012). However, as already mentioned in chapter 1.2, a distinct classification by means of these pallets is anything but simple. Therefore, the establishment of tools for molecular taxonomic species identification provide a valuable method for a fast, inexpensive and reliable identification. In order to be able to determine the occurring wood-boring animals according to the DNA barcoding approach (Hebert *et al.*, 2003) it was necessary to develop a new COI primer pair specific to *T. navalis*. This step was essential, as the universal primers of Folmer *et al.* (1994), which are usually used in the animal kingdom, have not yielded any us-

able results. This was rather surprising as these primers usually yield promising results with a wide range of different animal groups such as fish, insects or even other marine invertebrates. They also work well with other teredinids for which COI sequences have been successfully generated (Borges *et al.*, 2012; Shipway *et al.*, 2014). The difficulties of 'barcoding' *T. navalis* can be seen in the lack of COI sequences in the relevant databases, e.g. GenBank, and were also reported by other research groups (L. Borges, pers. com.). There is no information available about possible reasons so that may only be speculated about them. The most likely explanation is a significant difference between primer sequence and target sequence consequently not allowing for a sequence match. Such mismatches are known for many marine metazoans and resulted in a modified version of the Folmer primers showing improved consensus with many groups of marine invertebrates (Geller *et al.*, 2013). With the newly established COI primers (*cf.* chapter 3.1), developed before the redesigned primers of Geller *et al.* (2013) were created, it was possible to process both *T. navalis* and other teredinids.

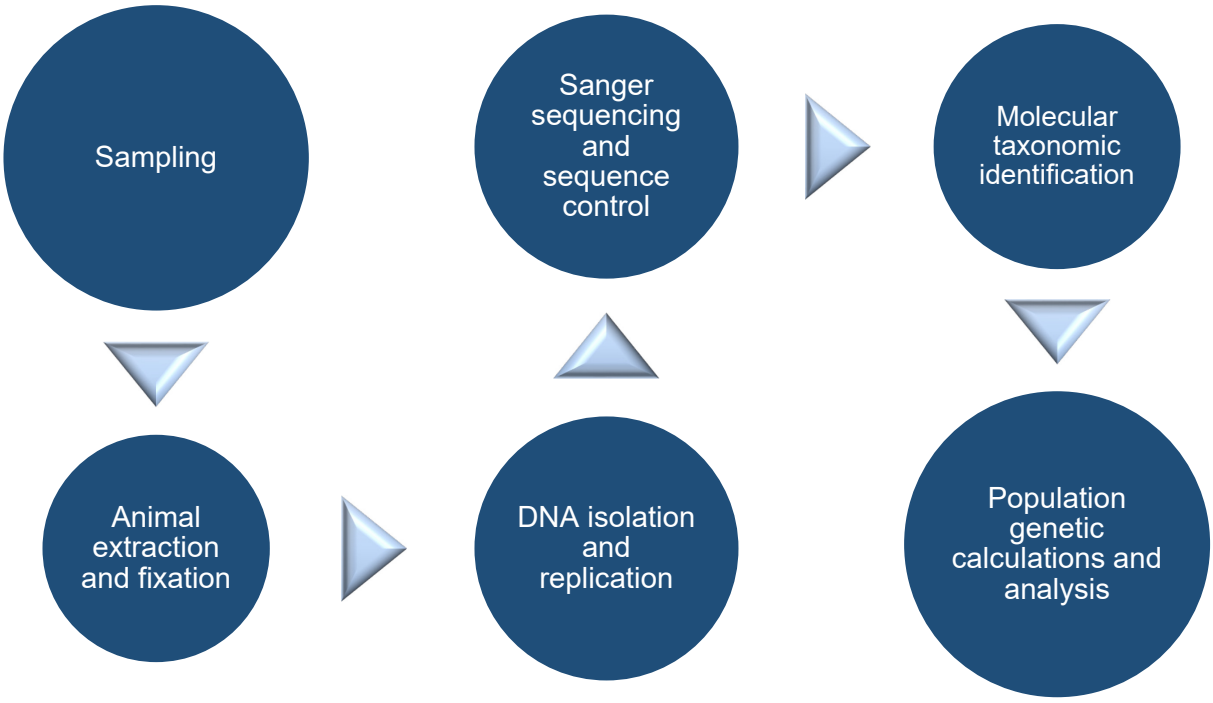

Figure 13 | Flowchart for the processing from the animal to the subsequent population genetic analysis. The size of the circles corresponds to the estimated amount of work required for each step within this thesis.

Molecular taxonomic identification of *Teredo navalis*

The analysis of all specimens investigated in this thesis showed that *T. navalis* was present at all sampling sites. The samples from Brittany, the Mediterranean Sea and the North American East Coast (DNA isolates) had already been identified as *T. navalis* using classical taxonomy. This identification was now confirmed via COI DNA barcoding. For the North Sea, the detection of *T. navalis* as the only shipworm species found during the present examinations was

surprising as generally several shipworm species like *L. pedicellatus, N. norvagica,* and *P. megotara* have been reported by various authors (e.g. Turner, 1966; Borges *et al.*, 2014b). One possible interpretation could be that, at least in previous studies, misidentifications might have occurred since only morphological taxonomic identification methods were applied.
Nevertheless, it is also conceivable that *T. navalis* has a higher ecological tolerance regarding the prevailing environmental conditions in contrast to other shipworm species and may have found more suitable living conditions within the anthropogenic influenced sampling sites. Furthermore, it is also possible that *T. navalis*, due to its high reproductive potential, was more abundant than other species in these areas and was therefore collected with a greater likelihood.

DNA barcoding approach

The samples which were investigated for this thesis mainly originated from the North Sea and the Baltic Sea but also from Brittany, the Mediterranean Sea and the North American East Coast. The determined COI sequences consisted of 700 to 750 base pairs each. The final alignments displayed of 675 base pairs without gaps or missing data. However, since COI sequences of *T. navalis* had not been available in the relevant databases until the beginning of this thesis, it was not possible to successfully perform a molecular taxonomic identification. Therefore, and to compare the present results with those of an earlier phylogenetic work of Distel *et al.* (2011), the 18S and 28S sequences of some specimens were additionally determined. At the beginning of the present investigation, these sequences were the only ones available in GenBank. To illustrate the relationship of specimens from America and Europe, a combined dataset with the sequences of Distel *et al.* (2011) was created (Fig. 2 in chapter 3.1).
The analysis of these sequences yielded similar results compared to the previous study. The combined 18S/28S data set were identical, showing no differences between the specimens sampled on both sides of the Atlantic. This is also reflected in the phylogenetic tree calculation, which included only a single sequence of *T. navalis*. Within the phylogenetic maximum likelihood tree, *T. navalis* grouped perfectly into the Teredinidae and appears as a sister group of the *Lyrodus pedicellatus* complex (Fig. 2 in chapter 3.1). This is well supported by a bootstrap value (probability value) of 99 %. These facts demonstrate that *T. navalis* is a species with an amphi-atlantic distribution. This is not surprising as almost all authors state a worldwide distribution of this species (e.g. Turner, 1966; Nair & Saraswathy, 1971; Borges *et al*., 2014b).
By comparing the recently acquired 18S/28S sequence data with those of Distel *et al.* (2011), and a simultaneous classical taxonomic determination of the pallets of some selected speci-

mens of the Baltic Sea, the COI sequence data of this thesis could be unambiguously assigned to the species *T. navalis*. All animals analyzed in the course of this work were identified by this approach. For the first time, this method enabled the molecular taxonomic identification of this species by using the DNA barcoding approach. Finally, both DNA datasets presented in this thesis genetically confirms the amphi-atlantic distribution of *T. navalis*.

Occurrence of *Teredo navalis* in the Baltic Sea

All samples from the Baltic Proper were exclusively assigned to only one species, *T. navalis*. Conversely, these results regarding the verification of *T. navalis* mean that the changes concerning the population permanence in the Baltic Sea since 1993 are not due to a new shipworm species in the investigated area. This corresponds to older studies (Nakel, 1954; Hahn, 1956) as well as recent reports on this region (Sordyl *et al.*, 1998; Borges *et al.*, 2014b). Appelqvist *et al*. (2015b) reported *T. navalis* to be the most abundant teredinid species in western Swedish waters, the entrance area of the Baltic Sea, together with only one other known shipworm species (*Psiloteredo megotara*). However, the abundances of the latter species are very low with only a few observations of single individuals (Norman, 1977; Appelqvist *et al.*, 2015b). Norman (1977) and Appelqvist *et al.* (2015b) uniformly reported the sampling site Mölle in the Kattegat as the southern boundary of the occurrence of *P. megotara*. These two studies, in conjunction with the results from the present thesis, showed that currently only one shipworm species exists in the Baltic Proper.

Occurrence of a sibling species

Sibling species are common and occur in all major marine groups and habitats (Knowlton, 1993). In the North Sea and Baltic Sea region, the two species complexes of *Marenzelleria spp*. (polychaetes; Bastrop *et al*., 1998) and *Mytilus spp*. (mussels; Steinert *et al*., 2012) are particularly well known. Although the phenomenon of sibling species can be observed in marine shipworms, there are no such signs in the case of *T. navalis*. Since the intra-species variance of the species with respect to the COI locus is 0.19 % (K2P, pairwise distances), the value is well below the, however not very sharp, 2.2 % threshold to distinguish species (Ratnasingham & Hebert, 2013). Therefore, the presence of sibling species of *T. navalis*, at least for the investigated area, can be excluded.

The COI sequences did also not show a difference between specimens of *T. navalis* from Europe and North America, which strongly supports the hypothesis of a single species on both sides of the Atlantic Ocean. This is also reflected in the determined first median-joining network analysis (Fig. 3 of chapter 3.1). It is evident that two sequences of the North American East Coast represent the central haplotype of the network. This dominant central haplotype is

thus present at all sampling sites, meaning there are no differences in this COI sequence on either side of the Atlantic. This allows further conclusions, which will be explained in the following paragraphs.

4.2 Population genetic calculations and analyses

Collapsed populations and undiscovered cryptic populations

Since the DNA barcoding approach was used to demonstrate that only *T. navalis* occurs in the southern Baltic Sea, this species was examined in more detail with regard to its present distribution compared to past distribution patterns. Especially since little is known about the occurrence of the species between the various documented mass outbreaks. One reason might be that the population repeatedly collapsed and completely disappeared from the southern Baltic Sea. This may be caused by the low salinity in the Baltic Sea, which makes reproduction impossible and thus repeatedly leads to the collapse of the population (Sordyl *et al.*, 1998). If the living conditions following the mass outbreaks in the preceding decades were not favorable for reproduction, the life span of 2 - 3 years could explain the duration of the mass outbreaks of *T. navalis*.

Otherwise, the existence of several small cryptic populations in the entrance area of the Baltic Proper is conceivable, that may have had remained undiscovered until a new outbreak due to improved living conditions (Sordyl *et al*., 1998). However, where exactly these populations might be located at is completely unknown. At this point, it can be hypothesized that larvae from the North Sea could repeatedly be introduced either by water currents or by ballast water (Sordyl *et al*., 1998) and have thus always caused new mass outbreaks. To address whether these past mass outbreaks are reflected in the population structure of *T. navalis*, various analyses of the genetic population structure in the North Sea and Baltic Sea were carried out.

Genetic diversity

For the genetic analyses, 23 specimens from each of the 13 sampling sites (Fig. 11) were examined, ranging from Brittany in the West to the island of Rügen in the East. The general population genetic indices as the total number of haplotypes (H), the haplotype diversity (Hd), the nucleotide diversity (π) and the number of average nucleotide differences (K) for all sampling sites were calculated. The Hd values of all analyses carried out in this thesis range from a minimum of 0.692 to a maximum of 0.913 (Tab. 2 in chapter 3.2). These are generally valid values for Bivalvia (e.g., Mao *et al*., 2011; Li *et al*., 2015).

In contrast, the values of the haplotype diversity of the individual sampling sites appear to be high compared to the work of Lasota *et al*. (2016) on *Mya arenaria* that also included sites in

the North and the Baltic Sea (Hd values between 0.441 and 0.699). The latter authors attribute this to a bottleneck effect after the Pleistocene glaciation. Such a bottleneck or founder effect can therefore be ruled out to *T. navalis* due to the results shown here.

Comparing the COI data sets of chapter 3.1 and 3.2 of this thesis, the total Hd values are approximately equal with 0.771 (n = 172) and 0.776 (n = 299). At this point, it is noteworthy that the calculation in chapter 3.2 shows a higher Hd value for the Baltic Sea compared to the North Sea (Tab. 2 in chapter 3.2). This is unusual and hardly known for any other bivalve species in the Baltic Sea (Johannesson & André, 2006). In addition, the two sampling sites revealing the lowest haplotype diversity (Hd = 0.692; Emden, Kristineberg) are located in the North Sea while the highest haplotype diversity (Hd = 0.913; Dranske) was found in the Baltic Sea. Remarkably, Dranske is also the most eastern sampling site in the study area.

This is uncommon for brackish waters like the Baltic Sea. As a marginal sea, there is only limited water exchange with the North Sea and a strong salinity gradient from West to East. This leads to a minimum of species diversity around salinity values of 5 - 7, as neither full marine species nor species primarily inhabiting freshwater are provided optimal living conditions (Snoeijs-Leijonmalm, 2017). Moreover, decreasing abundances along the salinity gradient may result in an increased genetic drift. This often leads to a loss of genetic diversity of populations within the Baltic Sea compared to populations of the same species outside. Johannesson & André (2006) have described this in a species-by-species meta-analysis for several taxa in the Baltic Sea. *Macoma balthica* presents the only exception they found that was by 30 % genetically more diverse in the Baltic Sea compared to the North Sea. However, this seems to be due to two genetically different lineages, one Atlantic line in the western and one Pacific line in the eastern Baltic Sea (Nikula *et al.*, 2008). By contrast, the results of the present thesis exclude two different genetic lineages of *T. navalis*. Additionally, there is no reduction in genetic diversity detectable along the salinity gradient up to the distribution boundary. Various authors (Johannesson & André, 2006; Johannesson *et al*., 2011) ascertained that there usually is a sharp distinction of the genetic population structure of a species inside and outside the Baltic Sea. For *T. navalis*, however, there is no sharp biogeographical break between the North Sea and the Baltic Sea and no loss of genetic diversity can be observed. Since the abundances of *T. navalis* are also decreasing along the salinity gradient towards the East, there must be other reasons for this phenomenon. The enormous genetic diversity could be attributed, among other things, to the high reproductive potential and ongoing gene flow, e.g. by larval drift. Moreover, the number of different haplotypes might be too high so that the total number was not represented by the maximum number of samples (n = 23) per sampling site. An increase in the number of samples could consequently result in an adjustment of haplotype diversity between the different locations and thus also between the Baltic Sea and the

North Sea. Mainly as haplotype diversity is positively correlated with the number of individuals sampled per location and the number of locations surveyed in a study (Muirhead *et al*., 2008).

Genetic population structure analyses

The high genetic diversity of *T. navalis* is also reflected in two phylogenetic network analyses (Fig. 3 in chapter 3.1, Fig. 2 in chapter 3.2) of the COI locus. Both median-joining networks display a similar star-shaped structure with the same central haplotype in the center. The haplotype Tn 1 occurs most frequently and is present in both networks at all locations examined. The proportion of private haplotypes occurring only at one sampling site is almost identical in both networks (75.0 %, n = 172; 72.6 %, n = 299), even if the sample size differs considerably. Both the shape of the networks and the high proportion of private haplotypes are clear signs of an expanding population. It is likely that all private haplotypes originated from the predominant ancestral haplotype Tn 1 after the expected sudden population expansion. A similar star-like phylogeny of COI haplotypes is also known for the bivalve mussel *M. arenaria* (Lasota *et al*., 2016). This could be due to a similar way of life as *M. arenaria* is also a eurytopic organism with a comparatively long pelagic larval stage of three weeks (Lasota *et al*., 2016).

In this case, however, these two networks cannot identify geographical distribution patterns and the presence of certain clades. These findings were underlined by the determined F_{ST} values and the calculated AMOVA analysis. Both calculations clearly show that no differentiated populations can be detected in the area under investigation. The calculated pairwise F_{ST} values ranged from -0.0150 to 0.0172 and were not significant for any sampling site (Tab. 3 in chapter 3.2). The difference between the locations is small as the values deviate far from one, which would mean complete differentiation (Wright, 1978). Therefore, it is not possible to identify a genetic population structure with the determined F_{ST} values of pairwise differences. The hierarchical AMOVA analysis also explains the majority of genetic variability (99.96 %) within the various locations but not between them. Even a differentiation between North Sea and Baltic Sea is very limited with a value of only 0.12 % (Tab. 4 in chapter 3.2).

Thus, all data of the present work indicate that there are no genetically differentiated populations of *T. navalis* in Central Europe. Such a limited genetic population structure is common and can be observed for other marine Bivalvia in Europe, such as the soft-shell clam *M. arenaria* (Lasota *et al*., 2004; Strasser & Barber, 2009). Nevertheless, this is in strong contrast to some other marine invertebrates of European waters like amphipods or polychaetes. Their populations are strongly structured showing different genetic lineages due to the survival in different glacial refuge (e.g. Bastrop *et al*., 1998; Krebes *et al.*, 2011). This is mainly due to

the lack of planktonic larval stages and a limited gene flow caused by reduced reproduction compared to *T. navalis*.

Similarly, there is no evidence of a genetically independent or isolated Baltic Sea population. All signs of the population genetic calculations point to a Central European panmictic population between Brittany and the eastern distribution boundary in the Baltic Sea. However, it seems unlikely that individuals of all sites interbreed with the same probability as they are up to 3900 km apart. The unrestricted migration of the larvae prevents a greater differentiation of the individual locations from each other resulting in genetic homogeneity. Since limited gene flow can contribute to genetic population structuring (Sá-Pinto *et al*., 2012) the opposite seems to be the case in the context of this thesis. Though, a lack of population structure of a species over several hundred kilometers is not unusual in marine Bivalvia as they are often characterized by a frequent gene flow between the locations (Vierna *et al*., 2012, and references therein).

Larval dispersal

At this point, both the high reproductive potential and the widespread drifting of larvae come into play. As a classic r-strategist, the high number of offspring ensures that at least some larvae find a suitable piece of wood for colonization. In this regard, short-term larviparous shipworms outperform other reproduction modes and therefore show the highest abundances (Turner, 1966). This can be seen in fully developed planktotrophic larvae showing a higher ability to survive long dispersals. Since the retention time in the water column can last up to 34 days (Nair & Saraswathy, 1971), the larvae can spread long distances through water currents. Depending on the velocity of the water currents, larvae can be dispersed over several hundred kilometers within a few weeks and even seem to be able to cross ocean basins (Scheltema, 1971). According to a biophysical model of the Baltic Sea developed by Appelqvist *et al*. (2015a), larvae of *T. navalis* may spread over a maximum of 400 km during one reproduction season. This dispersal appears to be an essential factor for the spatial distribution and the genetic connection between the otherwise geographically isolated populations (Scheltema, 1971). The importance of oceanographic connectivity for the population structure of marine invertebrates with pelagic larval stages has been shown by various authors (e.g., Seebens *et al*., 2013; Wrange *et al*., 2016). In this context, oceanographic connectivity seems to have a greater influence on the regional genetic population structure than the geographical distance between the individual sites (Wrange *et al*., 2016).

Therefore, other factors also need to be considered. Previous studies on marine invertebrates have shown that anthropogenic vectors can improve the spread of species over long distances (Wrange *et al*., 2016). Theoretically, anthropogenic influences such as transport via ballast

water could also contribute to a homogeneous distribution of *T. navalis* larvae in different biogeographic regions. In addition, the fact of the 13 sampling sites being located in high traffic areas (e.g. the Kadetrinne in the Baltic Sea is crossed by an average of 50,000 vessels annually; WSA, 2014) and the aforementioned occurrence of *T. navalis* larvae in ballast water (Gollasch, 2002) can contribute to the distribution of teredinid species (Borges *et al*., 2012, 2014b; Voight, 2015). The transport of larvae in ballast water, possibly against predominant main currents (Seebens *et al*., 2013), could be responsible for larval dispersal across biogeographical boundaries (Canales-Aguirre *et al*., 2015) that may ultimately lead to an increased gene flow.

As a second factor, adult teredinids may also use driftwood as a transport vector to travel long distances (Nair & Saraswathy, 1971; Scheltema, 1971). This is the case in large parts of the tropical and subtropical regions leading to specialists such as the aforementioned species *Uperotus clava*. Although the amount of natural driftwood in Central European waters is limited and reliable statistics do not exist, the total sum of wood in the North Sea and Baltic Sea would be sufficient to play a role as a distribution vector.

In this context, it is even more important to gain a deeper understanding of the distribution history of this species in Central European waters in order to be able to make predictions of possible range expansions in the future.

Demographic expansion

For the analysis of demographic history, the sampling sites between Brittany and Dranske were considered a panmictic population. The results of these calculations are shown in detail in chapter 3.2 (Fig. 4, Tab. 5). All indications of these calculations point to a sudden demographic expansion after a historical population decrease event. Whether this is due to a bottleneck effect or a founder effect cannot be conclusively clarified with the present study.

In this context, a possible starting time of the sudden demographic expansion was also calculated. Depending on different substitution rates of the COI locus for Bivalvia and an assumed generation time of one per year, the calculated starting time dates back to approximately 55,556 to 111,111 years ago. In addition, the geographical starting point of this expansion could also not be determined with the data presented here, as the preliminary extent of sampling data does not suffice for a global coverage. However, due to the last glacial period, which ended about 10,000 years ago in Europe, this place is most likely not located in Central Europe. At that time, the Baltic Sea and large parts of the North Sea were covered with glaciers (e.g. Grunewald & Scheithauer, 2010) and are therefore not considered a refuge for surviving populations. Some authors even assume a glacial ice shield up to the Iberian Peninsula (e.g. Grunewald & Scheithauer, 2010). Hence, in Europe, only the Mediterranean Sea or an

area south-west of the Iberian Peninsula could be a hypothetical starting point of this sudden expansion. Thus, the assumption that the place of origin of *T. navalis* is to be found in the estuaries of the great European rivers (Schütz, 1961) should be put into question.

Additionally, two more theories exist that allocate the place of origin to either the region of the subcontinent India (Hill & Kofoid, 1927), or the Caribbean region (Moll, 1914). At first glance, both theories seem to be more reasonable as there are distinct mangrove forests in both regions, which are undoubtedly considered shipworm habitats. The validation of either theory, however, cannot be conclusively clarified in the context of this thesis. Voight (2015) speculated that *T. navalis* has repeatedly been introduced in different areas, both temperate and tropical areas, and consequently, an original area cannot be determined. Nevertheless, there is a high probability that *T. navalis* originated in full marine waters. This could be drawn from the fact that the species showed faster growth in test panels exposed for about the same time period (June - November 2012) in the more saline North Sea (approx. 4 - 5 cm) compared to the Baltic Sea (approx. 1 - 2 cm). Under full marine conditions, the species seems to be closer to its salinity optimum in terms of the growth rate. Since the origin of *T. navalis* is still unclear, the species must furthermore be considered cryptogenic according to the definition of Carlton (1996).

Likewise, both the number of shipworm species and their abundances are significantly higher in tropical regions as mentioned before. The number of teredinids from mangrove regions could range from 11 species in Brazil (Baretto *et al.*, 2000) to up to 23 at the east coast of India (Nair & Saraswathy, 1971). Roughly a third of all known shipworm species occur in this habitat (Voight, 2015). Compared to the temperate zone, higher numbers of natural wood resources providing habitats, such as mangrove roots and fallen trees washed into the water from the rainforest, could explain this distribution pattern (Moll, 1940; Roch, 1955).

The almost hermetic sealing of the tubes through the pallets, which all teredinids species are capable of, could be an indication of a co-evolutionary development. As mangroves can only be found in the tropical tidal areas, it would have been essential for the wood-boring mussel species to develop a protective mechanism against the regularly recurring low tide conditions.

Even though the first reported mangrove fossils from the early Cretaceous are somewhat younger than the first teredinid fossils that date back to the Jurassic, it appears more than likely that the worldwide distribution of mangroves in all tropical regions also led to the successful distribution of the Teredinidae.

Historical distribution

The global distribution resulted in the fact that, as world trade has been increasing rapidly since the 15th century, teredinids have been registered in regions such as some parts of Europe

where they had not been present before. Consequently, the great seafaring nations of the 15th, 16th and 17th centuries that sent their explorers and business travelers on many different shipping routes around the globe were particularly affected. Therefore, the interest in the wood-destroying organisms in Europe grew again with the strong increase of active trading between Europe and the rest of the known world. Many expeditions using wooden ships had to be postponed or canceled, such as journeys from discoverers like William Dampier, Francis Drake (Moll, 1914) or Christopher Columbus. Judging from a travel report that Columbus had written to the Spanish king before his return from his fourth voyage in 1503, he seemingly had to go ashore in what is now Jamaica as three of his ships were “drilled and eaten up by the *Teredo*” (Moll, 1928).

Problems with shipworms were also reported when local wood was used for permanent structures in the water, as it had been common practice in the past centuries. Probably one of the best-known examples, as briefly mentioned in the introduction, is presented by the great flood in the Netherlands due to the breakage of wooden dyke gates in 1731. The trigger of this severe infestation with shipworms was a drought following a period of hot and dry summers, which resulted in a reduced freshwater outflow in this region. This caused an increase of salinity of inland waters followed by heavy mass occurrences of a teredinid species that seems to have been introduced to this area at that time (Voight, 2015). By the infestation with shipworms, most likely *T. navalis*, several parts of dyke constructions were severely damaged and collapsed eventually. The event went down in history to even appear in the German saying "*Holland in Not*" that originated from that time (Hahn, 1956).

Therefore, it is likely that the first settlement of *T. navalis* was located in Central Europe along the coast of the Netherlands. The Netherlands were one of the leading colonial nations in Europe during the 17th and 18th centuries and one of the world's leading global trading nations. Their enormous fleet enabled them to establish trade relations with numerous colonies overseas. These colonies were primarily located in Asia (mainly in regions of today’s India, Indonesia and Sri Lanka), America (partly North America but mainly Central America such as the Dutch Antilles) and Africa. All these regions could have been potential source habitats of *T. navalis* due to their geographic location. Consequently, it is likely that the species first settlement within Central Europe took place in the Netherlands during this highly active trading period. Especially since one infested wooden ship is sufficient to transport millions of shipworms across the oceans (Moll, 1940).

As mentioned in the introduction, the first confirmed report from the Mediterranean dates back to 1792 and thus some decades later than the first documentations for Central Europe. This could be an indication that the colonization of Europe by *T. navalis* started at an initial point in the Netherlands to a successively range expansion to adjacent waters.

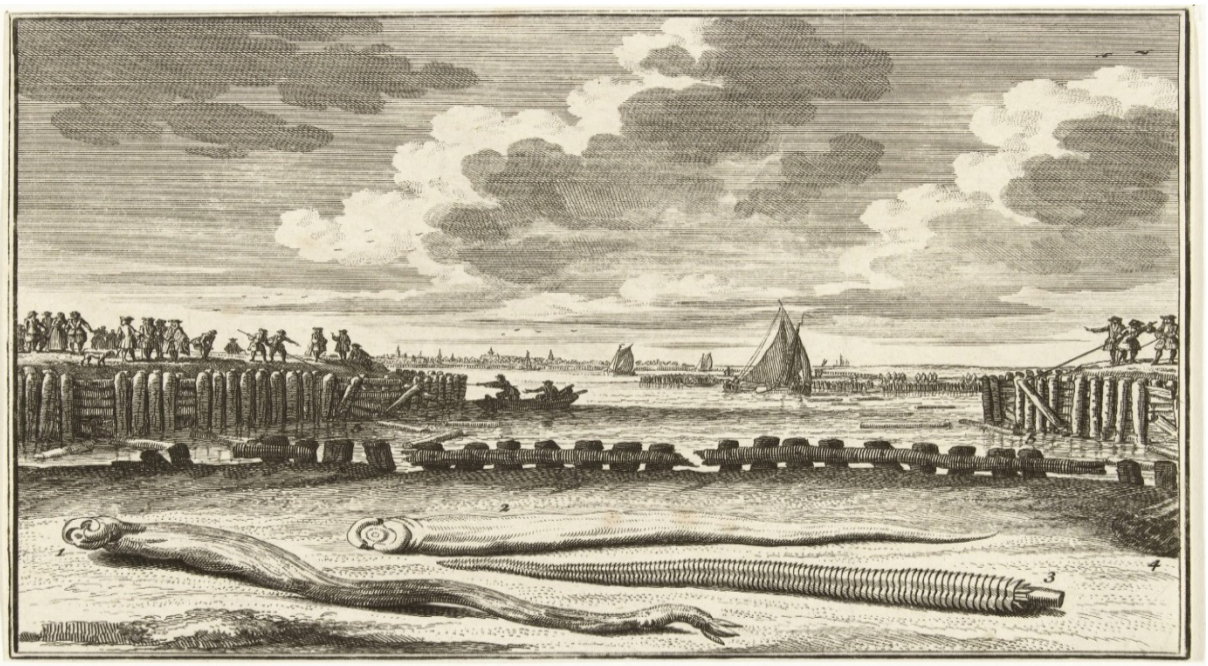

Figure 14 | Shipworms affecting wooden dike constructions, 1731 (Abraham Zeeman, 1731-1733; Rijksmuseum Amsterdam).

Natural range expansion

The fact that probably several entry events by numerous ships have taken place to introduce great quantities of larvae could explain why no signs of bottleneck or founder effect can be found in the genetic data. Since *T. navalis* has a great reproductive potential it is of course possible that billions of larvae enter a new habitat at once. It is indeed a special feature that only larvae will be introduced due to the sessile adult stadium. The great success of colonization might also be attributed to the species tolerance towards broad ranges of temperature, salinity and oxygen content (Roch, 1932). Since *T. navalis*, as a brackish water tolerating species, may not have had competition by other teredinids populating available wood resources in the introduced region (Borges *et al*., 2014b), the species might have spread relatively unhindered. Additionally, the short-term larviparous reproduction mode could have been of benefit here.

The next reports on *T. navalis* from Central Europe were mentioned approximately 100 years later in 1835 for the Baltic Sea. At this point, a successive range expansion from the North Sea in the area of the Netherlands to the adjacent waters seems to be the most likely explanation. The expansion potential of *T. navalis* proves very effective in the successful colonization

of new patchy habitats (Borges *et al*., 2014b) and the associated further expansion into new areas.

The finding that all specimens of the different sampling sites hardly genetically differ from each other strongly indicates that the animals originate from a single source population. This former founder population of Central Europe might presumably be located in the Netherlands. Due to the long pelagic larval stadium, it seems likely that a natural range expansion into the Baltic Sea via the Skagerrak, the Kattegat and through the Danish Straits has taken place. Such natural range expansions into the Baltic Sea are well known from other marine organisms, e.g. for the polychaete *Marenzelleria viridis* (Essink & Kleef, 1993; Blank *et al.*, 2008) or the dinoflagellate *Prorocentrum minimum* (Hajdu *et al*., 2000).

It is quite possible that *T. navalis* used different wooden structures as stepping stones for a successive expansion. In addition, wooden resources appear sufficiently available in the transition area from the North Sea to the Baltic Sea, both in earlier times and nowadays, as a foundation for settlement. In addition, due to the high reproductive potential and the long retention time of the larvae in the water column, the probability of finding a suitable piece of wood for settling seems to be increased. This is proven by the heavily infested test panels, which were exposed at the different sampling sites in the North Sea, Beltsee and Baltic Sea. All deployed wooden panels showed high rates of shipworm infestations within the different years, even though the exact numbers of boreholes were not determined in cases when it was only a matter of obtaining genetic samples in some years. Therefore, using a more comprehensive sampling, abundances of *T. navalis* in the southern Baltic Sea were determined in order to ascertain whether there is a continuous rang expansion and where the current distribution boundary is located.

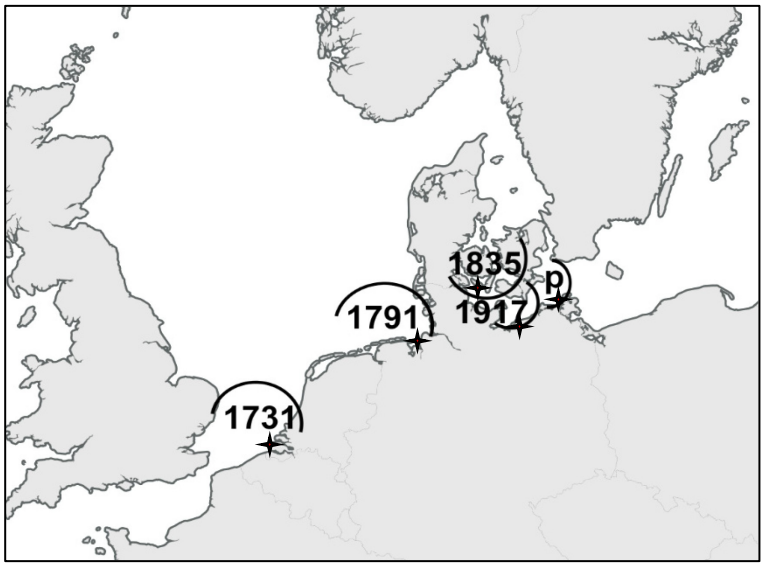

Figure 15 | Years of confirmed sightings of *Teredo navalis* in different regions of the North Sea and Baltic Sea. p: present distribution boundary.

4.3 Impact of salinity and temperature on abundances and distribution of *Teredo navalis*

Infestation of coastal protection structures by *Teredo navalis*

The infestation of coastal protection structures at the southern German Baltic Sea coast can be traced back to the year 1917 (Cordshagen, 1964). Local wood (e.g. pine) had traditionally been used for hydraulic engineering for many years before, which indicates that wood-boring species had only marginally or not at all occurred in this region before 1917 (Cordshagen, 1964). There may be several reasons for this. First, shipworms may have occurred only in small quantities and were not detected as they did not cause any major damage. Secondly, at that time there was potentially not enough wood as habitat available. Thirdly, and most likely, a reason may have been the prior absence and first appearance of shipworms during the process of range expansion from the North Sea into the Baltic Sea. It was shown that *T. navalis* is able to tolerate low salinities and also colonize brackish waters instead of full marine sites unlike most other shipworm species. This is reflected in the wide tolerance of *T. navalis* towards salinity and temperature in contrast to other shipworm species such as *P. megotara* or *T. dominicensis* (Borges *et al*., 2014b). An indication of this might be the occurrence of various wood-boring bivalve species in the entrance area of the Baltic Sea. For the Skagerrak region, in addition to the presence of two other wood-boring bivalves (*P. megotara, N. norvagica*) the wood-boring isopod *Limnoria lignorum* is also known to occur here (Borges *et al*., 2014a). Further south, in the Kattegat and Belt Sea region, at least only one other representative of wood-boring organisms is present (*P. megotara*) (Appelqvist *et al.*, 2015b). For the Baltic Proper, on the other hand, there have always been reports of only one wood-boring bivalve species.

The current situation at the German Baltic Sea coast, with a permanently established *T. navalis* population since 1993, raises discussions about the likelihood of a further species expansion into areas of even lower salinities. As North Sea and Baltic Sea are currently lacking natural wooden sources, except for some examples when wood washed into the sea after coastal erosion, *T. navalis* is largely dependent on artificially introduced wooden structures. Therefore, the costs for maintenance of wooden structures are rising in regions of shipworm occurrences.

Due to its texture, strength, longevity and availability, wood is still widely used in hydraulic engineering. It is used in the construction of marinas, as mooring pile in maritime shipping and, what is more, as coastal protection measures, namely groynes, for shore stabilization. Many other materials such as plastic, concrete or metal had been tested in the construction of the groynes but with unsatisfying material characteristics (M. Bugenhagen, StALU MM; pers. com.). In addition, the HELSINKI COMMISSION (HELCOM RECOMMENDATION 16/3)

recommends that only natural materials such as wood are to be preferred for coastal protection measures in the Baltic Sea. The attempt to fend off shipworms by covering the wooden groyne piles with geotextiles, as tested by the University of Rostock, proved laborious, expensive and hence not suitable for large-scale use.

In recent years, groynes at the Mecklenburg-Western Pomeranian coast were usually made of local pine and provided an ideal habitat for *T. navalis*. They were strongly affected which resulted in very high maintenance costs within the last few decades. This is mainly because since 1993 the groynes made of pine had only remained intact for 2 - 3 years when severely infested by shipworms, compared to 30 - 40 years previously without infestation (M. Bugenhagen, StALU MM; pers. com.). In the areas where heavy infestation is to be expected, the groyne piles have to be made of FSC-certified sustainably produced tropical hardwood. Due to its solid structure and the alkaloids contained therein, it is less prone to be colonized by marine shipworms (Bavendamm & Roch, 1970). Since the infestation of this hardwood is much lower, it is to be expected that hardwood groynes will have a similar lifespan as the pine groynes without shipworm infestation and hence justify the higher acquisition costs (M. Bugenhagen, StALU MM; pers. com.). To reduce high acquisition costs and CO_2 emissions due to long-distance transports to a minimum, this hardwood is only used where shipworm infestation is most likely to occur. For all other locations, the favorable local pinewood is preferred.

Therefore, it is of immense importance to know what the present distribution situation looks like in order to be able to predict possible spreading scenarios. For this reason, an annual monitoring has been taken place, which has been carried out by various institutes in a sufficiently consistent manner since 1995. In 2012, this project was taken over by the University of Rostock while roughly the same sampling sites were examined. The sampling plan hardly changed and was only moderately adjusted over the years to conduct a systematic study. In particular, the influence of the two key factors salinity and temperature on the larval dynamics and on the present distribution boundary were extensively studied. Details are given in the material and methods section of chapter 3.3.

Borehole abundances

The two key factors salinity and temperature and the combination of both are of particular importance for the colonization of new habitats by shipworms. For the Danish Isefjord, Kristensen (1969) showed that *T. navalis* could tolerate lower salinities at higher temperatures. This finding of coupled temperature-salinity effects affecting the growth are also known for other teredinids such as *L. pedicellatus* (Eckelbarger & Reish, 1972). In this regard *T. navalis* shows the widest tolerance for salinity and temperature compared to other teredinid species

(e.g. *L. pedicellatus* I/II) investigated by Borges *et al*. (2014b). Appelqvist *et al.* (2015a) discovered that favorable conditions for *T. navalis* are always controlled by temperature when salinity is above 8. This threshold value was determined around the two sites Dranske (average salinity of 8.8) and Glowe, east of Hiddensee, and thus at the eastern boundary of the area under investigation. This was also the lowest mean salinity value measured over the entire period (Tab. 1 in chapter 3.3). At both locations, only few boreholes were discovered during the regular sampling from 2012 - 2015 (≤ 1 dm^{-2}).

In contrast, the borehole abundances at the other locations investigated were very high ranging between 90 (2015) and 990 (2013) boreholes dm^{-2} at a maximum. This is quite high compared to other locations within the entrance area of the Baltic Sea. Kristensen (1979) detected only a maximum of 27 specimens dm^{-2}, while Norman (1976a, 1977) and Appelqvist (2015b) reported abundances between 100 to 200 specimens dm^{-2}. While the number of boreholes was determined by x-ray examination for the latter studies, the number of boreholes was counted manually using stereomicroscope in this thesis. This method can lead to an overestimation of the successful settlement attempts since not every borehole visible from the outside needs to contain a living individual. Control counts of living individuals showed a successful settling of approximately 70 % of the larvae in relation to the total number of boreholes counted previously (own results, not shown here). The mean values of the borehole abundance per sampling site and year are shown in Figure 5 of chapter 3.3.

Similarly, a distinct tendency in the dependence of borehole abundances with respect to the decreasing salinity gradient to the east could not be shown for the years under investigation. In 2013, for example, the borehole abundance at the eastern, and thus less saline, sampling site Graal-Müritz was more than twice as high compared to the sampling site of Kühlungsborn (Fig. 5 in chapter 3.3). However, the intensity of infestations also varies greatly from year to year at the different locations. In Graal-Müritz for instance, the infestation fluctuates between more than 800 boreholes dm^{-2} in 2013 and only 40 boreholes dm^{-2} in 2015. This could assumedly be explained by different temperature regimes in the individual years since reproduction, survival and metamorphosis of the larvae are temperature-dependent (Nair & Saraswathy, 1971; Hoagland, 1986).

Time of settlement of larvae

An obvious tendency of the settling time of the larvae for the period under investigation could also not be seen. Settlement over the various years did not start, as expected due to the prevailing water temperatures, by the end of June but in most years only in mid-August or even late August. In two years (2012, 2014) the settlement at some locations (Boltenhagen, Kühlungsborn, Graal-Müritz, Zingst) lasted until October (Fig. 6 in chapter 3.3). Thus, the

settlement period did not start until 5 to 12 weeks after the presumed trigger temperature of 15 °C had been reached. Due to these different time intervals in the respective years, a clear trend for beginning and end of the main settlement period cannot be discerned.

Paalvast & van der Velde (2011a) also identified a late settlement at the port of Rotterdam from August to September although the temperatures suitable for reproduction had already reached in April and May. Kristensen (1979) determined a settling period in the Danish Isefjord from mid-August to the end of September. In contrast, Appelqvist & Havenhand (2016) identified a settlement at the Swedish West Coast from the end of June to the beginning of July. These different starting times and the different lengths of the colonization periods suggest that apart from temperature, other factors also play a role in the successful settlement of larvae.

With regard to the two key factors salinity and temperature that are vital for successful colonization, no statistical correlation between the measured data and the determined larval abundances could be established in the present thesis. Other factors, such as the prevailing water and wind currents and substrate availability, seem to play a major role. Finally, the availability of wood in the respective regions and the associated retention time in the water column could have a major influence on the duration of the settlement period. However, these factors were not the subject of the present investigations and hence no statement can be made about their influence so far.

A peak in the settlement of larvae in connection with the end of a high-temperature period as determined by some authors (Ryabchikov & Nikolaeva, 1963; Kristensen, 1979) could not be proven within this thesis. Although the results suggest a tendency of intensive colonization after a longer period with high water temperatures, the gaps between sampling intervals of this thesis were too long to clarify this. Nevertheless, the presence of different ecotypes such as e.g. early or late spawners has hitherto been unknown for shipworms.

Influence of a Major Baltic Inflow on the occurrence of *Teredo navalis*

The mass occurrences of *T. navalis* appear conspicuously often in a temporal relation to a MBI into the Baltic Sea (Appelqvist, 2015). In the case of many non-indigenous marine organisms, mass occurrences were typically described some years after their introduction (Gollasch *et al.*, 2009), leading to their discovery in these regions.

Within this thesis, it could be demonstrated that, contrary to previous opinions, a MBI does not have a major impact on the abundance of *T. navalis* in the southern Baltic Sea. Although a brief increase in salinity was recorded at the sampling sites Boltenhagen, Kühlungsborn and Dranske, this did not appear to have any impact on larval dynamics. In contrast, the borehole abundances in 2015 after the MBI in 2014 were lower than they had been in previous years

(Fig. 5 chapter 3.3). Since the large salt water inflows usually take place in winter due to the necessary weather conditions, an influence on the larvae distribution seems to be uncertain as they are not present in the water column during this season. Nevertheless, increased salinities in the early summer months can lead to better reproductive conditions and thus increase larval production. However, this cannot be confirmed by the available data of the period under investigation.

Thus, it also seems questionable whether the MBI of 1993, as often stated, can actually be related to the subsequent mass occurrence of *T. navalis*. For some reasons this cannot be confirmed. First, the MBI had taken place in January 1993 (Dahlin *et al*., 1993) and hence long before the spawning period of *T. navalis* in these regions. Moreover, the phenomenon of countless washed up groyne piles to the shore already occurred in the late summer of 1993 (M. Bugenhagen, StALU MM; pers. com.). Even if the MBI improved reproductive conditions in the early summer subsequently followed by a mass occurrence, the time for this annual cohort would be too short to severely damage the groyne piles. Consequently, the infestation must have happened in the years before probably between 1990 - 1992. Even extremely severe attacks of shipworms do not lead to a destruction of groyne piles by waves until two to three years after the beginning of the attack. Consequently, there seems to be no connection between an MBI and a mass occurrence of *T. navalis* in the Baltic Sea.

Present distribution boundary and possible spreading scenarios

As mentioned before, the lowest salinity values of a four-year mean salinity of 8.8 were determined at the sampling site Dranske and the borehole abundances were very low compared to the other sampling sites. In addition, the boreholes usually did not contain living animals. However, the occasional occurrence of unusually favorable conditions, for example changing water currents in combination with an increased water temperature, could lead to more or less successful single larval settling events. This can also occur at locations where the species seems to be at its physiological limit since the lower physiological limit in terms of salinity is around 7. Nevertheless, the individuals are able to develop to the adult stadium at this salinity and growing to a length of more than 20 cm. In the case of the sampling site Dranske, this occurred only in small quantities (about two animals dm^{-2} in 2016, own obs.). This might indicate that Dranske is not yet the present distribution limit within the Baltic Sea. Whether the animals are able to reproduce under these conditions cannot be answered now because neither sexually mature animals nor larvae have been detected.

At the easternmost sampling site Glowe, only boreholes with dead larvae but no living animals were detected. According to the results of this thesis, the location Dranske must

therefore be regarded as the present eastern distribution boundary of *T. navalis* at the Baltic Sea coast of Mecklenburg Western-Pomerania.

Whether this will also apply to the future remains to be evaluated. There are unconfirmed recent reports of sightings of *T. navalis* at shipwrecks around the small island called Greifswalder Oie (T. Förster, German Oceanographic Museum, Stralsund; pers. com.). This area is located approximately 50 km to the southeast of Glowe and is estimated to have an average salinity of 8. This suggests that this species has spread to the east of Rügen and would be the first sighting for this region, if confirmed.

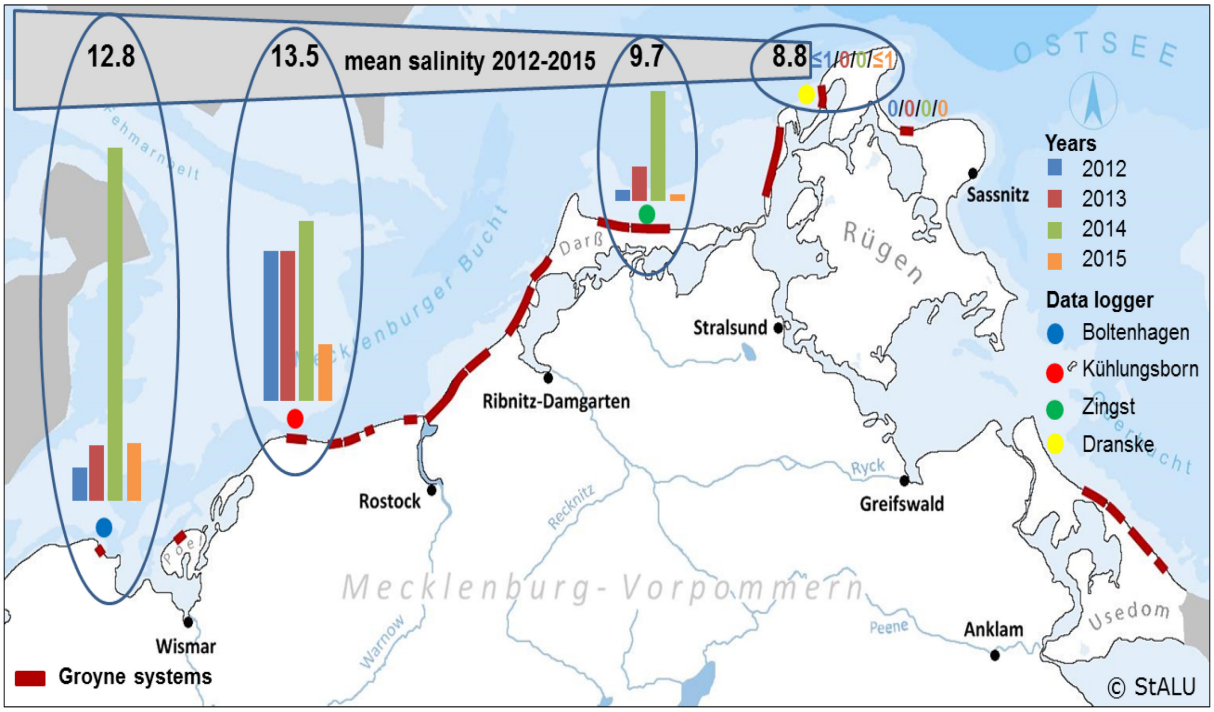

Figure 16 | Schematic illustration of mean abundances of boreholes per dm^2 test panel per year and positions of the autonomous data logger between 2012 - 2015. (Details in the result section in chapter 3.3.)

Other unconfirmed reports also mention an infestation of shipwrecks in the Gdañsk Bay (I. Pomian, National Maritime Museum, Gdansk; pers. com.). This sighting seems to be even more remarkable as the Gdańsk Bay is about 300 km further east of Rügen. The seawater surface salinity of approx. 6 - 7 in this area is likely too low for successful growth and reproduction of *T. navalis*. Nevertheless, it is conceivable that there may have been a spread of larvae with the deeper, salt-rich water currents into this area (Fig. 17). There is an average surface water salinity of 7 to 8 up to about the southern tip of Gotland as can be seen in Figure 17. Considering the bottom water salinity is higher, the possible colonization range is even larger and reaches the Finnish south coast (Fig. 17). If so, the potential distribution area of *T. navalis* in the Baltic Sea would be considerably larger than the previously populated area.

At this point, the MBIs of the last few years could have played a decisive role if the inflow was strong enough to cross the Darss Sill. However, caution should always be exercised when

dealing with reports like these without proof of living animals. These observations could represent old boreholes, as the ship may have already been infested before sinking. In order to exclude this possibility, a thorough examination by experienced specialists is required. Therefore, a closer cooperation with underwater archaeologists could be advantageous for future investigations of *T. navalis* in the Baltic Sea.

A report from 2003 shows that settlement of shipworm larvae in this eastern region is indeed possible (Olsen, 2003). However, this report by a Danish engineering company is only available in the national language and does not meet scientific requirements in its implementation. This is mainly due to the lack of replicates and a poor documentation. From November 2001 to November 2002, pine (*Pinus sylvestris*) test panels have been exposed at a depth of approximately 50 cm below the water surface in the port of Rønne on Bornholm Island. This site is located about 100 km east of Rügen. During the investigation period, salinities between 5.9 and 7.9 were measured. Unfortunately, additional information on the detected animals (e.g. pictures of pallets) and methods of identification are not available. Nevertheless, the test panels were subjected to an x-ray examination. Thus, it seems to be undisputed that an infestation with shipworms was detected, most likely with *T. navalis*. Although it cannot be completely ruled out that this could have also been *N. norvagica* or *P. megotara*, so far both species have only been reported further north at higher salinities. Nevertheless, this is, remarkably, the first proof of the presence of a shipworm species in this region. For the above-mentioned reasons it seems essential to carry out further investigations of both the surface water around Bornholm and the adjacent deep water. In this case, sampling far away from the coast could be of particular interest to find the larvae that may drift within the deeper water currents.

For the southern Baltic Sea, the data of this thesis, however, showed no changes in the distribution of *T. navalis* over the past decades compared to the work of Sordyl *et al*. (1998). The occasional slight shifts of the distribution boundary of an animal species at the edge of its distribution are seemingly common. This has already been observed for *T. navalis* in earlier times. Reports of the 1950s indicate a spread of this shipworm species in the Baltic Sea limited to the virtual line Sassnitz - Trelleborg (Nakel, 1954). The later investigation by Sordyl *et al*. (1998) could only prove a distribution up to the island of Hiddensee that is situated further to the West. In this respect, the determined occurrences in this thesis seem to be within an assumed fluctuation range of a species at the edge of its distribution depending to the combination of the prevailing temperature and salinity conditions.

However, in the face of climate change, this present distribution might change significantly and may result in a range expansion of *T. navalis* into areas of the Baltic Sea that have not been infested so far. Although, Appelqvist *et al*. (2015b) has not yet confirmed a range expansion of *T. navalis* at the Swedish West Coast in recent years from 1973 to 2008 it appears to

be a possible scenario. Borges *et al*. (2014b) predicted probable locations that could potentially be populated by the species as an outcome of their modeling calculations. The latter authors forecast the spread of *T. navalis* into the eastern Baltic Sea and discuss a possible adaptation to lower salinity conditions. Though, this assumption is based on the occurrence of *T. navalis* in the Baltic Sea at a salinity of 7.

This cannot be confirmed with this thesis. As shown in chapter 3.3, the assumption of the latter authors is presumably based on an incorrect location of some exposed test panels that have been included in the calculation and hence without any foundation in this regard. As the larval distribution within deep water currents has not yet been investigated, this cannot be answered at the moment and requires further investigations. However, the water temperature of the Baltic Sea has already increased in recent years (~ 1.3 °C between 1985 - 2005; Mackenzie & Schiedek, 2007) and *T. navalis,* as mentioned above, might tolerate lower salinities in this respect. Therefore, these areas further in the East must not be lost out of sight and have to be taken into account in future investigations and monitoring programs.

Most recent climate projections agree that the surface water temperature of the Baltic Sea will increase in the near future and sea-ice covers will decrease significantly (BACC II, 2015). Predictions of whether salinity values will increase or decrease are still inaccurate due to the different calculation models. Given the expected increase in precipitation and the associated increase in fresh water input from rivers, the trend is towards a decreasing salinity for the Baltic Sea in the wake of climate change (HELCOM, 2013; BACC II, 2015). Only the study of Hansson *et al*. (2011) predicts an increase in salinity in a warmer climate. The latter authors suggested that the salinity in the Baltic Sea would increase as a result of reduced fresh water inflow through rivers. This does not, however, equally apply to all areas and will lead to regional differentiation. There will presumably be more rainfall in the northern Baltic Sea region, while less precipitation is expected in the south. Furthermore, Hansson *et al*. (2011) assume that the reduced fresh water supply in the south has a greater influence than the increase in the north and the Baltic Sea as a whole will thus become more saline.

Hence, a general warming combined with local reductions of salinity in different regions of the Baltic Sea appears to be the most likely scenario. Due to the great inaccuracies in the hydrological models, no conclusions can be drawn concerning changes in salt water transport (BACC II, 2015). If these projections are confirmed, it is likely that the distribution of *T. navalis* will change either very slightly or not at all. Although the species can tolerate lower salinities at higher temperatures, the simultaneous occurrence of salinity reductions could prevent spreading.

However, a presumably decreasing salinity will play a greater role in possible dispersals compared to rising temperatures since the adaption to salinity changes is more strenuous than

adapting to changing temperatures (Holopainen *et al*., 2016). However, rising temperatures can lead to an extension of the growth season of invertebrates (Holopainen *et al*., 2016) and with that to a larger dispersion potential.

Nonetheless, more detailed physiological investigations have not been carried out recently for *T. navalis*. Such information is lacking, in particular with regard to the effects of a possible acidification of the Baltic Sea, which could have serious consequences for calcifying organisms such as bivalve mollusks. Since key processes such as growth and reproduction are influenced, this can also affect abundances, diversity and distribution of a species (HELCOM, 2013).

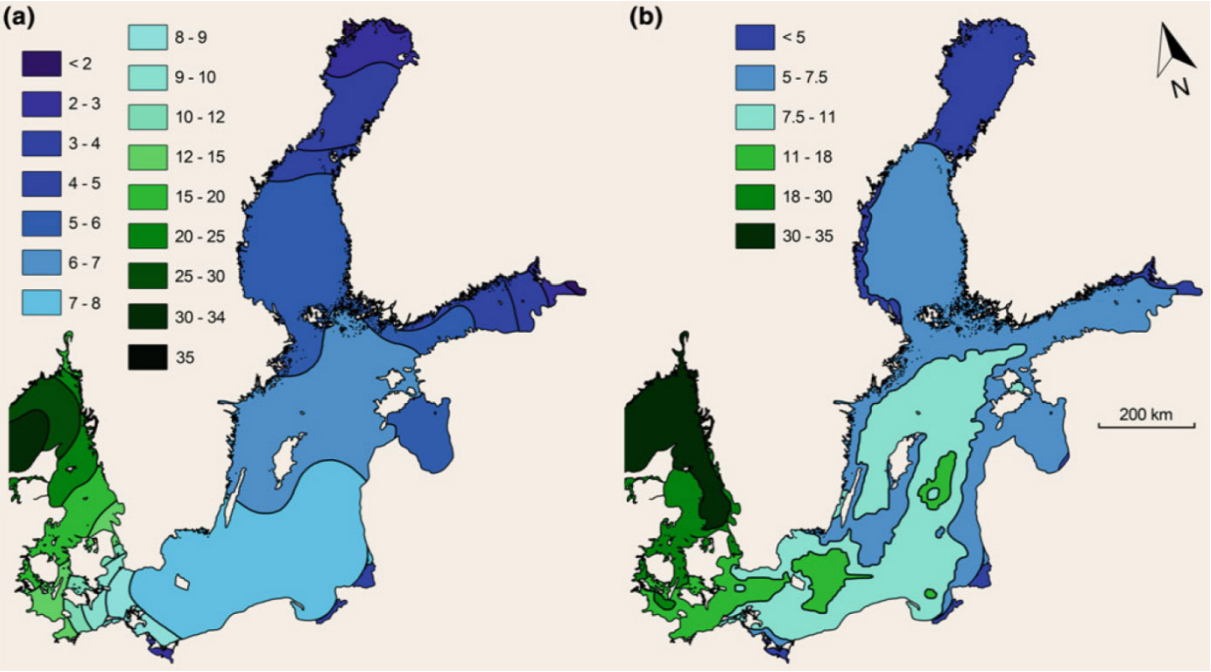

Figure 17 | Schematic illustration of the average sea surface (a) and deep bottom (b) salinity in the Baltic Sea. © Snoeijs-Leijonmalm, 2017

Summing up, since the last and still ongoing mass outbreak of *T. navalis* in 1993, there has apparently not been a substantial change in the distribution of this species in the southern Baltic Sea, since no extensive range expansion to the east but only slight shifts of the distribution boundary have been observed.

4.4 Conclusions and directions of future research

This thesis attempted to clarify the present taxonomic status of wood-boring bivalves in the southern Baltic Sea, the possibility of a genetically differentiated Baltic Sea shipworm population and the present distribution boundary of the occurring shipworm species in comparison to the first detection of a permanent and self-reproducing population in the southern Baltic Sea in 1993.

To this end, the present distribution situation and the genetic population structure of *T. navalis* in European waters have been comprehensively investigated. A specific primer pair for the COI locus has been developed to provide a simple and cost-effective molecular taxonomic tool for the reliable identification of *T. navalis*. Finally, it has been shown that there is, at present, only one shipworm species in the southern Baltic Sea, *T. navalis*, which had been mentioned there earlier. The subsequent classification into the phylogenetic system of bivalves and further phylogenetic calculations also excluded the existence of a sibling species for the area under investigation.

Furthermore, no genetically isolated Baltic Sea population could be identified as none of the sampling sites are genetically differentiated from the others. On the contrary, it is indeed the case that all signs point to a widely connected, panmictic population.

In contrast to the decreasing abundances of boreholes along the salinity gradient, no decrease of genetic diversity in eastern direction has been observed. In some cases, even higher haplotype diversities were observed within the Baltic Sea compared to the North Sea. This is unusual for a presumably marine species in a brackish water environment. In addition, due to the high genetic diversity of all sampling sites, there are no signs of a founder effect or bottleneck effect for the area under investigation. The calculated phylogenetic networks also reflect this. Due to their star-shape, and in connection with the results of the other population genetic calculations, a sudden demographic expansion of *T. navalis* in Europe can be suggested. Therefore, all data indicate a natural range expansion of *T. navalis* in Central European waters from only one source population. Since the first scientific evidence for Central Europe reported from today's Netherlands in 1731, the starting point for the subsequent range expansion seems to be there. At present, it cannot be conclusively clarified whether this has happened by a single or several different introduction events, as could have been assumed due to the trading frequency of wooden ships at that time. However, it could not be finally ascertained within this thesis whether there might have also been secondary introduction events by ships into the Baltic Sea or whether continuous reintroductions with larvae from the North Sea occurred. In order to gain a deeper understanding of the settlement history in Europe and the subsequent spread of *T. navalis*, a comprehensive worldwide sampling and the processing of the samples with highly polymorphic markers such as microsatellites seems to be essential.

Since it has been shown that there is only a single species of shipworms in the Baltic Proper, an attempt was made to determine whether a change in distribution could be demonstrated compared to previous investigations. For this purpose, the periods of colonization as well as the larval abundances of *T. navalis* were determined. Simultaneously recorded data of salinity and temperature were used to detect correlations between these two important key parameters and the occurrence of *T. navalis*. Due to the large variances of borehole abundances both in

the different years and between the different locations, no definitive statement could be made regarding these factors influencing the settlement. Perhaps the prevailing wind and water currents are responsible for a successful settling to a greater extent than previously thought. Nevertheless, the present distribution boundary of *T. navalis* at the Mecklenburg-Western Pomeranian coast could be determined. The island of Hiddensee was the easternmost sampling site, which revealed high rates of infestation and living larvae in some of the test panels. At the sampling site Dranske, living animals were rarely detected and at the easternmost location Glowe only occasionally boreholes without living animals have been found. Therefore, the present distribution boundary in the coastal waters seems to be between the sampling sites Dranske and Glowe and thus in a region with a salinity of around 8 to 9. Consequently, no range expansion of this species could be determined in the investigated area for the last decades. However, since there are occasional reports of sightings located further east, these locations should in any case be sampled in the context of a subsequent monitoring.

For predicting possible future dispersal scenarios, it is crucial to identify where the larvae that settle at the coast of Mecklenburg-Western Pomerania originate. In addition, if all groyne fields were made of sustainable tropical hardwood, that is hardly infested, and if natural wooden resources were scarce, where would the refuge areas of sexually mature adults be found? Conversely, does this imply that larvae are potentially introduced from areas that have not yet been identified? Alternatively, are there possibly only relatively small numbers of parent animals in a few wood sources that, due to their high reproductive potential, are sufficient to produce these large numbers of larvae?

These questions are interesting for future research activities and require clarification. Sampling of the planktonic larvae at different times during the spawning period and at different locations should also be considered. For this purpose, it might be interesting to apply test panels at sea signs of maritime shipping, as they are more distant from the coast. If the spawning sources could be detected, it would be easier to forecast various dispersal scenarios by using different hydrographical models. This would make it possible to identify potentially affected areas and use sustainable tropical hardwood or, if possible, stones for coastal protection structures.

In addition, the complex and expensive attempt to remove (all) wooden sources from the system could also be launched, thereby depriving the habitat of *T. navalis* in the Baltic Sea. The question remains, however, whether such an initiative is realistically feasible, that has already been considered by Appelqvist *et al.* (2015a).

It would involve enormous coordination efforts in several countries with a very uncertain outcome. The attempt to identify all natural and anthropogenic sources of wood is likely to take several years. Such an approach would require a strong political interest in order to be provid-

ed with the necessary financial resources. This is hampered by the fact that the Baltic Sea is one of the most wreck-rich waters in the world. Estimates predict up to 100,000 wrecks in the entire Baltic Sea. Although not all of them are known in detail yet, a major amount will most likely consist of wood. Since many of these wrecks cannot be salvaged due to their conservation status and limited financial resources, it is impossible to remove this food source of *T. navalis* from the system.

For a deeper understanding, the analysis of prevailing wind and water current conditions during the time of the spawning period of *T. navalis* also seems essential. In addition to the further analysis of salinity and temperature - and climate change considered - it also seems necessary to observe additional factors playing a role for *T. navalis* development, such as the pH value. In this context, a further examination of symbiotic bacteria might also be of interest. Under changing environmental conditions, they could play a decisive role in the future distribution of *T. navalis* in the Baltic Sea. This might be in the form of better adapted bacterial strains of *T. turnerae* or possibly also by colonization of *T. navalis* by completely different bacterial species.

With this thesis, the basis for such further work has been created.

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6 Specific contributions to the publications

I)

Weigelt, R., Lippert, H., Borges, L., Appelqvist, C., Karsten, U., Bastrop, R. (2016). First time DNA barcoding of the common shipworm *Teredo navalis* Linnaeus 1758 (Mollusca: Bivalvia: Teredinidae): Molecular-taxonomic investigation and identification of a widespread wood-borer. J Exp Mar Biol Ecol 475, 154-162. doi:10.1016/j.jembe. 2015.11.008.

Authors contributions: **RW** and RB collected the samples. **RW** and RB developed the primers and acquired the sequence data. All authors interpreted the data. **RW** drafted the first manuscript and all authors critically revised the work and approved the final version of the manuscript.

II)

Weigelt, R., Lippert, H., Karsten, U. and Bastrop, R. (2017). Genetic Population Structure and Demographic History of the Widespread Common Shipworm *Teredo navalis* Linnaeus 1758 (Mollusca: Bivalvia: Teredinidae) in European Waters Inferred from Mitochondrial COI Sequence Data. Front Mar Sci 4, 196. doi:10.3389/fmars.2017.00196.

Authors contributions: **RW**, HL, and RB collected the samples. **RW** and RB acquired the sequence data and all authors analyzed and interpreted the data. **RW** drafted the work and all authors critically revised the work and approved the final manuscript.

III)

Lippert, H., **Weigelt, R.**, Glaser, K., Krauss, R., Bastrop, R. and Karsten, U. (2017). *Teredo navalis* in the Baltic Sea: Larval Dynamics of an Invasive Wood-Boring Bivalve at the Edge of Its Distribution. Front Mar Sci 4, 331. doi:10.3389/fmars.2017.00331.

Authors contributions: HL has to a great extend performed the field and laboratory work, has analyzed the data and written the first draft of the manuscript. **RW** has taken part in the field and the laboratory work, has contributed to figure drafts and has edited the manuscript. RK has essentially contributed to field and laboratory work. RB has been involved in project development and has contributed to the manuscript. UK has significantly been involved in project development and realization and has essentially edited the manuscript. All authors have approved the final manuscript.

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Somit geht nun ein (langes) Kapitel zu Ende, doch ein neues ist bereits aufgeschlagen. Es bleibt so wie es immer war - spannend…

Declaration of authorship

Selbstständigkeitserklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende kumulative Dissertation selbständig angefertigt und ohne fremde Hilfe verfasst habe. Es wurden keine außer den von mir angegebenen Hilfsmitteln und Quellen verwendet und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen sind als solche kenntlich gemacht.

Rostock, den 19.10.2018 Dipl. Biol. Ronny Weigelt