Thin-sections of marine bivalve shells: a window to environmental reconstruction on a daily scale?

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Thin-sections of marine bivalve shells: a window to environmental reconstruction on a daily scale?

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Zusammenfassung

„Bioarchive“ sind Organismen, die während ihres Lebens Hartstrukturen bilden, die über den Tod des Organismus hinaus erhalten bleiben und aus deren anatomischer, morphologischer und chemischer Beschaffenheit Informationen über Umweltbedingungen zu Lebzeiten des Organismus gewonnen werden können. Beispiele für Bioarchive sind Krusten-Rotalgen (Skelette), Korallen (Skelette) und auch Muscheln, deren Schalen sich durch ihre sehr hohe Auflösung für Studien in der Sclerochronologie eignen. Im Allgemeinen beschäftigt sich diese Wissenschaft mit Wachstumsmustern und chemischen Beschaffenheit in Hartstrukturen der Bioarchive. Verschiedene Proxies, wie z.B. die Breite von Jahresinkrementen oder das Verhältnis von stabilen Sauerstoffisotopen ($\delta^{18}O$), können entschlüsselt werden, um die in den Hartschalen enthaltende Information zu „lesen“. Durch die weite geografische Verbreitung der Islandmuschel *Arctica islandica* und ihrer Langlebigkeit eignet sich diese besonders für solche Studien. Zuwachsraten, die anhand der Inkremente ausgemessen werden und die geochemische Eigenschaften des Schalenkarbonats geben Auskunft über Umweltfaktoren wie Wassertemperatur, Nahrung, Salinität und Wasserverschmutzung.

Einige Studien haben gezeigt, dass die tägliche Wachstumsrate in verschiedenen Mollusken im Verlaufe eines Jahres variiert. Schöne et al. (2005a) berichten, dass Mikroinkremente täglichen Zuwachs anzeigen. Mit Hilfe einer geeigneten Methode ist es mögliche die Breite dieser Mikroinkremente zu messen und daraus Rückschlüsse über Wachstumstrends und Klimarekonstruktion auf täglicher Basis zu erhalten.

Um diese Mikroinkremente zu visualisieren habe ich Dünnschliffe der marinen Muschelart *A. islandica* und der Süßwassermuschel *Unio* sp. angefertigt. Da zur Dünnschliffherstellung von Muschelschalen kein Standardprozedere existiert, war das Ziel dieser Arbeit, eine geeignete Methode zur Herstellung dieser zu etablieren. Hierzu wurden unter anderem verschiedene Ansätze zur Einbettung, Anätzen, Bleichen und Visualisieren von Mikroinkrementen getestet.

Tägliche Mikroinkremente sind mittels der Dünnschliffe in *A. islandica*, als auch in *Unio* sp. sichtbar. Die Mikroinkrementenbreiten in den Süßwassermuscheln sind hierbei wesentlich kleiner (durchschnittlich 1,5 µm) als die der Islandmuschel (durchschnittlich 12,5 µm), dennoch sind sie in *Unio* sp. deutlich sichtbarer und können durchgehend gemessen werden. Die Visualisierung der Mikroinkremente in *A. islandica* ist wesentlich schwieriger und bedarf weiterer Ansätze im Labor (siehe Outlook Kapitel). Mikroinkrementmessungen in der Süßwassermuschel *Unio* sp. zeigen dagegen ein großes Potential und können mit den hier beschriebenen Methoden zukünftig als potentieller Umweltproxy etabliert werden.

Abstract

"Bioarchives" are organisms, which form hard parts over the course of their lifetime that remain even after the death of the organism. Environmental conditions prevailed during the lifetime of the bioarchives can be approximated from anatomical, morphological and geochemical properties on the shell. For instance, shell growth rates constitute a "proxy" of general living conditions, oxygen isotope ratios ($\delta^{18}O$) are an established proxy of water temperature, and shell content of heavy metals or of organic constituents can be indicative specific pollution histories. Due to their high resolution, bivalve shells are well suited for sclerochronological studies. Generally, this science focuses on growth rates and chemical properties of hard parts. The ocean quahog *Arctica islandica* is suited as a bioarchive due to its broad geographic distribution and longevity.

This study looks at growth patterns in the shells of the bivalve *A. islandica* (marine) and *Unio* sp. (freshwater). The objective was to establish standard procedures for shell preparation to visualize shell increments formed on a daily basis ("microincrements").

In order to visualize microincrements thin-sections of the marine bivalve *A. islandica* and the freshwater bivalve *Unio* sp. were prepared. Therefore, different attempts for embedding, etching, bleaching and visualization were tested.

Microincrements are visible in thin-sections of both genera. The microincrements of the freshwater mussel *Unio* sp. are significantly smaller (1.5 µm on average) than those of *A. islandica* (12.5 µm on average). However, microincrements in *Unio* sp. are more easily recognizable and can be measured consecutively over a range of more than one year. The visualization of microincrements in *A. islandica* remained more challenging and therefore additional attempts such as bleaching, etching and additional visualization techniques were tested for their potential to improve the visualization of microincrements. The visualization of microincrements in *A. islandica* still needs further improvement before measured microincrement widths can be correlated to environmental data. However, *Unio* sp. seems to have great potential and can be used as a window to reconstruct environmental data on a daily scale in the future.

1 Introduction

The knowledge how ecosystems react to changing environmental conditions is essential for well-constrained predictive climate-models (Schöne, 2013).

Bioarchives like red-coralline algae (skeleton), corals (skeleton) and mollusks are living organisms which from hard parts during their lifetime (Marchitto et al., 2000). Anatomical, morphological and geochemical properties give information about environmental conditions prevailing during the lifetime of those bioarchives. They help to understand climate changes on time scales up to centuries with an annual to sub-annual resolution (Markwick, 2007). In the case of mollusks the hard parts are usually precipitated in the form of calcium carbonate. Shell growth rates constitute a "proxy" of general living conditions like salinity (Davis and Calabrese, 1964), water temperature (Kennish and Olsson, 1975) and food (Page and Hubbard, 1987). Oxygen isotope ratios ($\delta^{18}O$) are an established proxy of water temperature (Schöne et al., 2005b), and shell content of heavy metals or of organic constituents can be indicative specific pollution histories (Krause-Nehring et al., 2012). Proxies were used to encode the recorded climate data.

According to Boecker (2000) climate change is often modulated by seasonality changes in periodicities in the Earth's orbital elements. Other archives like sediment and ice cores also exist. Sediment cores are the climate archives that cover the longest time spans (up to millions of years), but they have limited temporal resolution of about a decade usually and down to a few years at best (e.g. Jiang et al., 2005; Eiríksson et al., 2006). Ice cores may span several hundreds of thousands of years, but have an annual solution at best. Hence both archive types hardly provide information on annual scales and not at all at sub-annual / seasonal variability. Consequently, they cannot give information about sub-annual and seasonal dynamics, but this is important for our understanding of past climate dynamics and for proper modeling of paleo and future climate states. This explains our need for archives with annual and better temporal resolution.

Analogous to sclerochronology, dentrochronologists use trees as archives. Schweingruber et al. (1991) have shown that tree rings are suitable proxies for summer air temperature and precipitation on land. Those proxies do not have a sufficient resolution to determine seasonal variability in environmental parameter (Schöne et al., 2005a). Further, data obtained from tree rings are summer-biased and do not provide information about marine settings. Due to short life spans microfossils (e.g. foraminifera), obtained from low resolution marine sediment cores do not provide information on annual or sub-annual scales (Schöne, 2005b).

The ocean quahog *Arctica islandica* is suited for sclerochronological studies due to its broad geographic distribution (Schöne et al., 2005b) and its longevity (Schöne, 2013). Several studies about shell growth on annual (e.g. Schöne et al. 2005; Schöne, 2013) and seasonal resolution describe the multitude of research possibilities on paleoclimate, water quality monitoring and ecology (c.f. Schöne, 2013). Seasonal resolution is a unique feature in bivalve shells. Here, by identifying and looking at so called microincrements, even a daily resolution can be achieved.

Schöne et al. (2005a) present a study about daily growth rates in *A. islandica*. However, little is known about shell growth on a daily scale due to missing appropriate visualizing techniques. Due to higher growth rates in the early stages of life of mollusks, microincrements are expected to be best visible in the earliest ontogenetic years (Cargnelli et al., 1999). Going from the umbonal area to the ventral margin and in the direction of growth the number of visible microincrements decreases. Daily growth increments are orientated parallel to the more prominent annual growth lines. Clark et al. (1975) demonstrate that shell growth and biomineralization processes are controlled by biological clocks. Dependent on the species they can take place on various time-scales. For two species of pectinids it is shown that they form growth lines on a daily periodicity.

The potential of master chronologies providing environmental information over hundreds or even thousands of years is described by Jones et al. (1989). For example, a 489-year marine master chronology was used to reconstruct the marine climate in the Irish Sea (Butler et al., 2010). Furthermore, statoliths in squids record their environment with daily precision (Arkhipkin, 2005). Goodwin et al. (2001) used stable oxygen isotope measurements in combination with microincrement widths to obtain paleoclimatic information on sub-weekly and sub-monthly scales. These examples show the importance of studies of bio archives on sub-annual levels.

1.1 Aims and Objectives

This study focuses on the questions if thin-sections are a suitable method for visualizing microincrements in marine and freshwater bivalve shells, how a standard procedure to prepare such thin-section can be established and if obtained microincrement measurements can be used as a window to reconstruct environmental parameters on a daily scale. In detail, the following issues and questions should be addressed and answered in this study:

- Is it possible to prepare thin-sections of marine shells of *Arctica islandica*?
- Is it possible to prepare thin-sections of freshwater bivalve of *Unio* sp.?
- How to establish a standard procedure to prepare thin-sections of bivalve shells?
- Are there any differences in the preparation of thin-sections between both species?
- Are thin-sections a suitable method for visualizing microincrements?
- Which microscope methods are the best suited for visualizing microincrements?
- Can bleaching and etching procedures help to visualize microincrements?
- Which growth trends can be seen within one year?
- Can microincrements be measured and correlated to environmental datasets?
- What is the difference of microincrement widths between species?

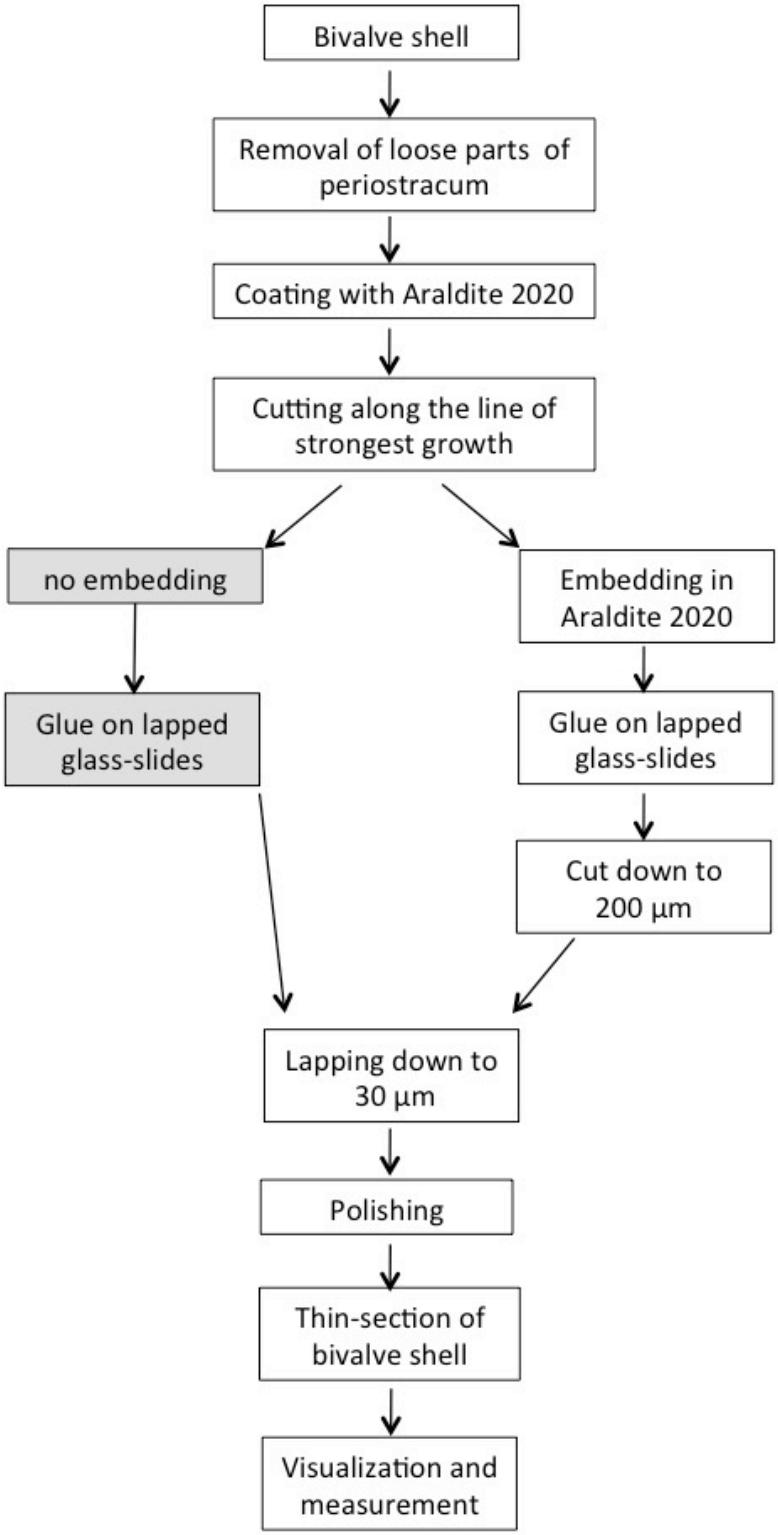


Fig. 1: Flow chart describing the preparation of thin-sections from bivalve shells. The grey-colored path has been tested, but finally considered unsuitable for the preparation of thin-sections.

2 Material and Methods

2.1 Marine bivalve Arctica islandica

The ocean quahog *Arctica islandica* is the "longest lived, non-colonial animal of the world" (Wanamaker et al., 2008).

2.1.1 Taxonomy

Table 1 shows the verified taxonomic range of *Arctica islandica*, which has previously been known as *Cyprina islandica*.

Table 1: Taxonomic range of *Arctica islandica* (http://www.itis.gov/, checked: 24.06.2014).

Kingdom	Animalia
Subkingdom	Bilateria
Phylum	Mollusca
Class	Bivalvia (Linnaeus, 1758)
Subclass	Heterodonta (Neumayr, 1884)
Order	Veneroida (H. Adams and A. Adams, 1856)
Superfamily	Arcticoidea (Newton, 1891)
Family	Arcticidae (Newton, 1891)
Genus	*Arctica* (Schumacher, 1817)
Species	*Arctica islandica* (Linnaeus, 1767)

2.1.2 Distribution

This species can be found in the temperate/boreal North Atlantic (Thórarinsdóttir and Einarsson, 1996), ranging from the Bay of Cadiz in Spain, north to Iceland in the northeast Atlantic, and from Cape Hatteras in North Carolina, USA, to the Canadian Arctic in the northwest Atlantic (Nicol, 1951; Merrill and Ropes, 1969; Abbott, 1974; Brey et al., 1990; Witbaard et al., 1999). Thereby, the water depth range of *A. islandica* varies between 10 – 280 m (Thompson et al., 1980a). Occasionally, the ocean quahog can be found in depths of 500 m (Nicol, 1951).

2.1.3 Physiology

The modern distribution for *A. islandica* implies a water temperature range from 1°-16°C (Golikov and Scarleto, 1973). Winter (1969) have demonstrated that the filtration rates are reduced by 50% when the temperature decreases from 12° to 4°C and respectively double when temperature increases from 4° to 14°C. *A. islandica* do not survive for more than a few hours at water temperatures below 0°C and consequently it is assumed a

boreal genus and not an arctic one (Nicol, 1951). Further, this species tolerates salinity ranges from 22 to 35 PSU (Winter, 1969).

Generally categorized as a suspension feeder (Cargnelli et al., 1999), Morton (2011) reclassifies the ocean quahog as a specialized deposit feeder (c.f. Schöne, 2013). Although the typical long siphons are missing, the sinking carbon from the suspended particles of epibenthic organic material, which characterizes the seabed and the rich surface water, is collected.

According to Lutz et al. (1981) *A. islandica* most commonly inhabits muddy and sandy sediments (Nicol, 1951), but also settles down to a wide array of other substrate types where it lives borrowed in the top 5 cm of the substrate (Morton, 2011). The main predators for young individuals of *A. islandica* are cod and lab. The mortality rate in medium sized specimens decreases and due to senility increases again for old animals (Brey et al., 1990).

2.1.4 Shell structure and biomineralization process

The surface of the outer shell layer of the ocean quahog *A. islandica* is covered by the periostracum, which protects the marine bivalve against dissolution as well as microbial attack (Wilbur and Saleuddin, 1983). The shell of *A. islandica* consists of calcium carbonate, which is largely aragonitic and structured in three layers: the outer and the inner shell layer as well as the myostracum. For sclerochronological studies the outer shell layer or the umbonal hinge plate are used. The shell is secreted by the mantle. In general, “the mantle and its outer epithelium, the periostracum and the interface between the outer epithelium, the periostracum and the growing shell” are necessary for the calcification process of the shell (Marin et al., 2012).

2.1.5 Annual and daily growth patterns

Annual and daily-scale growth patterns become visible in marine bivalve shells such as *A. islandica* due to interrupted growth. Moreover they do not grow at the same rate during their lifespan which explains more narrow annual increment widths in ontogenetic older shell portions. Growth rates decrease throughout the lifetime caused by biological aging. In general, variability in growth is a result from changes in physiology and environment (Schöne, 2013).

The distance of the alternating pattern of one thick growth line (GB1 = growth break) and one thinner one, GB2, is defined as an annual increment (Schöne et al., 2005a).

GB1 is formed in fall or early winter and is linked to the spawning cycle. Specimens belonging to one population are synchronous in forming GB1. During the period of formation of GB1 the rate of shell growth is relatively slow, whereas the growth rate is most rapidly in late spring and early summer. This concludes that the growth rates of *A. islandica* are not consistent throughout the year. This was also described by Schöne et al. (2005b). They could show that shell growth starts before winter minimum temperatures are reached and stops after the summer maximum. Moreover, shell growth is uniformly slow during the times with hottest and coldest seasonal temperatures (Jones, 1980, 1981; Thompson et al., 1980). Schöne et al. (2005a) suppose that the period of growth takes eight months in shells from the North Sea. Growing season ends in autumn / winter. As a conclusion, temperature seems to trigger shell growth either directly or indirectly. Thompson et al. (1980a) hypothesize that immature animals of *A. islandica* mimic the annual reproduction cycle. This would explain the interruption or at least the slow-down of shell growth and therefore the formation of growth rings.

2.1.6 Shell material

Variable numbers of shell of *A. islandica* from four different localities were chosen for the preparation of thin-sections.

Table 2: List of *Arctica islandica* specimens used in the study.

Sample ID	Country	Location	Date of death	Water depth [m]
090421	Germany	Helgoland	01 Aug 2005	40
090428	Germany	Helgoland	01 Aug 2005	40
090504	Germany	Helgoland	01 Aug 2005	40
Arc Is 316	Iceland	North East	2008	12
Arc Is 317	Iceland	North East	2008	12
Arc Is 331	Iceland	North East	2008	12
Arc Is 333	Iceland	North East	2008	12
Arc Is 345	Iceland	North East	2008	12
245625	Norway		2006	10-30
245629	Norway		2006	10-30
M3	USA	Maine	01 Jan 2010	ca. 40
T13	USA	New England	01 Jan 2010	ca. 72
V21	USA	Virginia	01 Jan 2010	ca. 80
W16	USA	New England	01 Dec 2009	ca. 46

2.2 Freshwater bivalve Unio *sp.*

Additionally, thin-sections of two freshwater shells of the genus *Unio* were prepared. Both have been collected from the Lago Maggiore, Italy.

2.2.1 Taxonomy

Table 3 shows the taxonomic range of *Unio* sp. There are several species of *Unio*, but the shell material used in this study has not determined up to the species level.

Table 3: Taxonomic range of *Unio* sp. (http://www.itis.gov/, checked: 12.07.2014).

Kingdom	Animalia
Subkingdom	Bilateria
Phylum	Mollusca
Class	Bivalvia (Linnaeus, 1758)
Subclass	Palaeoheterodonta (Newell, 1965)
Order	Unionoida (Stoliczka, 1871)
Family	Unionidae (Fleming, 1828)
Genus	*Unio*

2.2.2 Distribution

Worldwide more than 900 species of freshwater bivalves are described. They are found in streams, rivers and lakes. Bivalves of *Unio* can be found in Central Germany (Beierlein, 2011), the Netherlands (Versteegh et al., 2009), Scandinavia (Dunca et al., 2005) and in England (Negus, 1966; Dunca et al., 2005). Due to over-exploitation, environmental pollution (Bauer, 1988), habitat destruction (Gillies et al., 2003) and the introduction of invasive species (Burlakova et al., 2000; Klocker and Strayer, 2004; Riccardi et al., 1998) most species are today endangered of extinction.

2.2.3 Physiology

Unio sp. belonging to the order Unionoida tolerates an increase in salinity up to 3‰. Dettmann et al. (1999) and Versteegh et al. (2009) show evidence that growth of Unionidae bivalves stop below water temperature of approximately 12°C, while Yoshiumura et al. (2010) indicate a value of 10°C. Shell growth starts in April and stops in October showing that the growth season takes seven months (Dunca et al., 2005).

2.2.4 Shell material

Table 4: List of *Unio* sp. specimens used in this study.

Sample ID	Country	Location	Date of death	Water depth [m]
LB-LaMa-02R	Italy	Lago Maggiore	unknown	dead collected at beach
LB-LaMa-03R	Italy	Lago Maggiore	unknown	dead collected at beach

2.3 Localities

All *A. islandica* specimens in this study (Table 3) were collected alive at four different countries and respectively seven different localities (Fig. 2, Table 5).

Table 5: Geographical information on the marine bivalve shells of *A. islandica*. In total, 14 different shells from four different countries were used to prepare thin-sections.

Country/ Location	Latitude	Longitude
Helgoland	54°09.02'N	07.47.06'E
Iceland	66°01.68'N	14°50.96'W
Norway	56°09'N	11°48'E
USA		
Maine (M3)	43°75.0'N	68°30.0'W
New England (T13)	40°75.0'N	70°75.0'W
Virginia (V21)	38°30.0'N	74°00.0'W
New England (W16)	41°15.0'N	71°30.0'W

The following maps (Figs. 2, 3 and 4) illustrate the geographical positon of the different localities where the shells of *A. islandica* and *Unio* sp. has been collected.

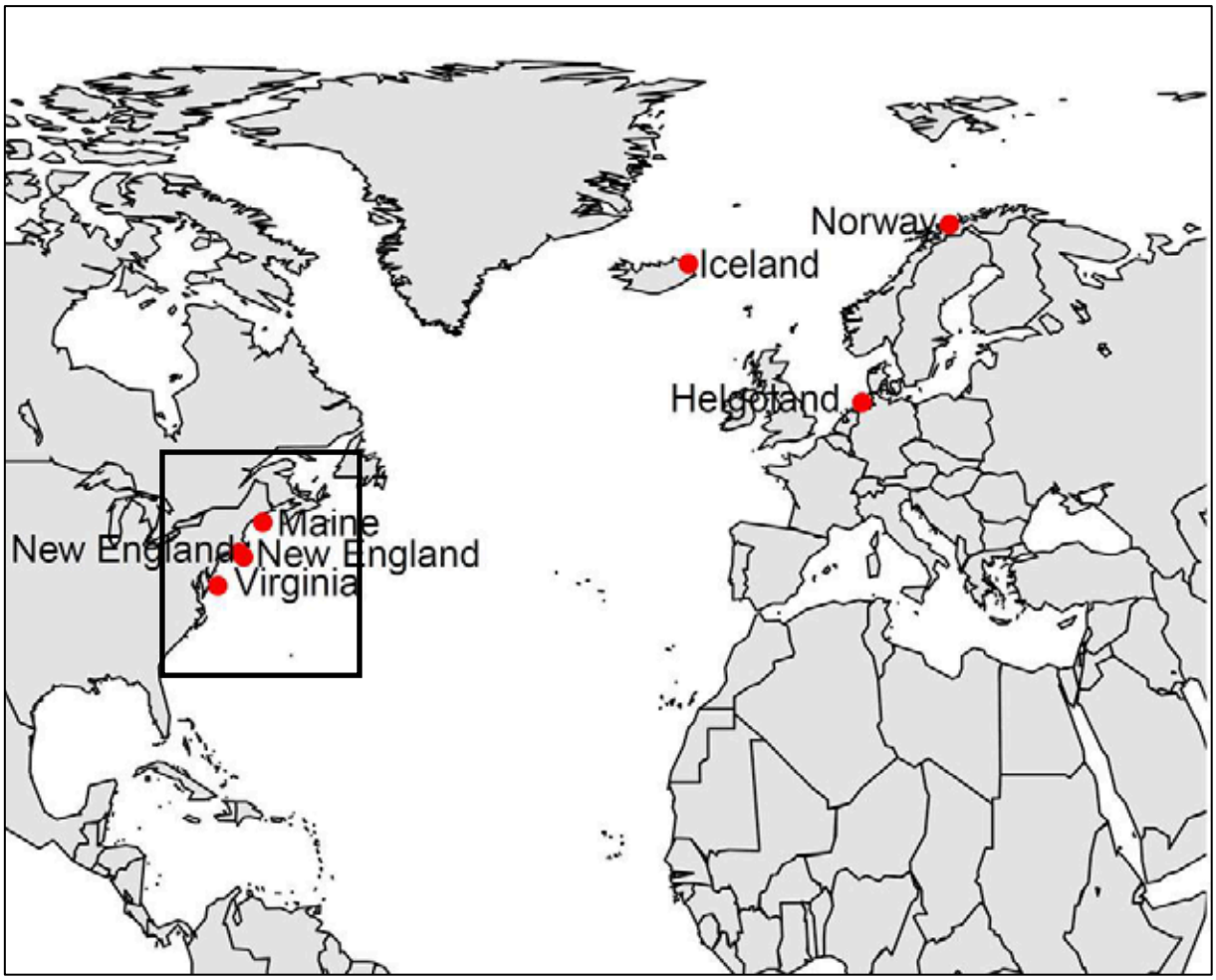


Fig. 2: Map of the localities where *A. islandica* has been collected.

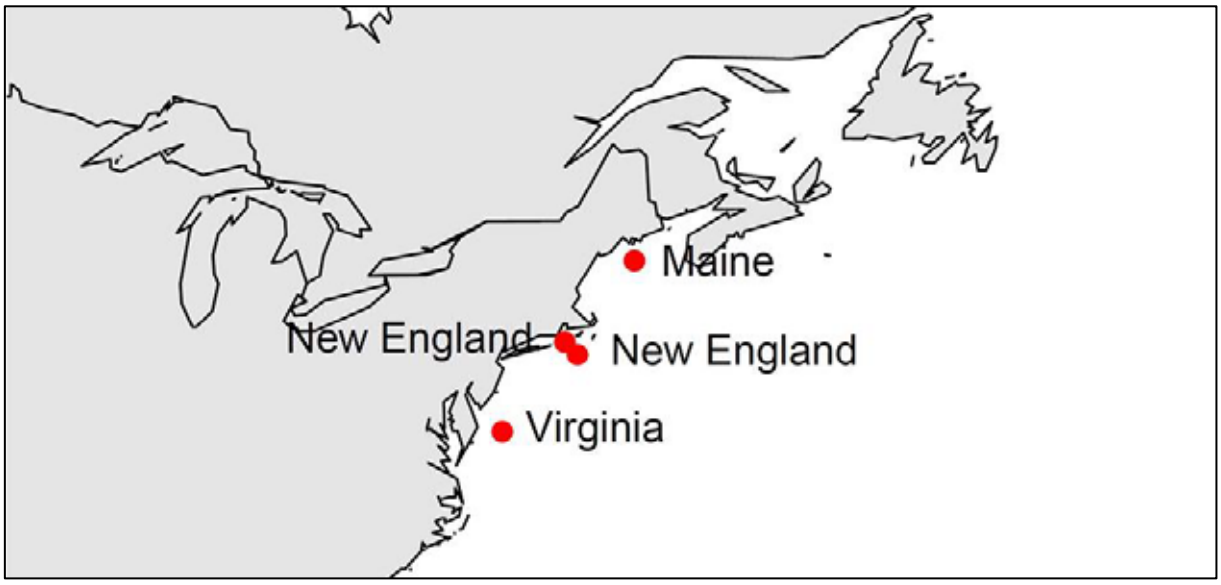


Fig. 3: More detailed view of the *A. islandica* localities in the USA as marked in Figure 2.

Specimens of *Unio* sp. were collected from the Lago Maggiore in Italy (Table 6).

Table 6: Geographical information about the Lago Maggiore locality, where the freshwater bivalve shells have been collected.

Country/ Location	Latitude	Longitude
Italy, Lago Maggiore	45°57.52'N	8°38.5'E

Fig. 4: Map illustrating the geographical position of the Lago Maggiore, which is located in the north of Italy. Illustrating the locality where the freshwater mussels *Unio* sp. were collected.

2. 4 Thin-sections of bivalve shells

I prepared thin-sections to visualize daily increments in bivalve shells. Several steps are required to visualize them. The idea of preparing a thin-section is that shell material is glued on a glass-slide and then lapped down to ~30 µm. However, on the micrometer scale glass-slides are not plane-parallel. Therefore, the glass-slides have to be lapped first. This is conducted to assure that they are equally thick throughout.

2.4.1 Lapping of glass-slides

The glass-slides are lapped at 60 rpm with the LOGITECH CL50 Compact 50 Lapping/ Polishing Machine (Fig. 5 (A)). Since the final thin-sections are approximately 30 µm thin, it is particularly important that the glass-slides are made plane-parallel, avoiding any inaccuracies, which would cause problems in the following process.

Firstly, 80 ml of LOGITECH Silicon Carbide Powder, 800 ml distilled water and half of the top of the anti-corrosion agent Corrozip-LF are mixed to an abrasive. To adjust the system for attaching the glass-slides a dial gauge (Fig. 5 (B)) is used. Thereby the undercut has to be considered, which is estimated as three times of the grain size of the LOGITECH Silicon Carbide Powder. This value of the undercut is reached after a lapping time of four to five hours. Using LOGITECH Silicon Carbide Powder with a grain size of 9 µm, the undercut was estimated to 18 to 20 µm by a lapping time of 30-60 minutes. Moreover it is very important to attach two similar formed glass-slides into the system to avoid irregularities caused during lapping. The mixed abrasive drops down to a rotating disc. The supporting head is on these disc and the glass-slides are hold by vacuum that is produced by a pump.

Afterwards the glass-slides as well as the supporting head of the lapping machine have to be cleaned before measuring to avoid any measuring errors. Any residues of the abrasive can cause deviations up to 9 µm. The thickness of the glass-slides has to be measured (see Fig. 5 (B) Dial gauge) to control if lapping was successful and to check if plane-parallelity has been achieved.

Fig. 5: (A) Lapping/ Polishing Machine and (B) Dial gauge.

2.4.2 Sample preparation

First of all, the shells are cleaned by removing sediment and loose parts of periostracum with a toothbrush to prevent that superglue drops off afterwards.

2.4.3 Coating

Afterwards, the shell has to be coated twice with Araldite 2020 to prevent the shell from breaking during cutting. Araldite has to set hard for 24 hours before it can be coated again.

2.4.4 Cutting

A first cut has to be done 1 cm left or right of the line of strongest growth to get a straight line which is necessary for fixing the shell material to a metal block. Therefore, the cut surface has to be grind with sandpaper with grain size of 20 µm to obtain a flat surface parallel to LSG (= line of longest growth). Following individual valves are fixed by Crystalbond 509 to a metal block. This metal block is the holder for the shell material during the cutting process (Fig. 6 (A)). It is screwed in the dedicated gadget and the shell material is cut along the LSG, which is perpendicular to the growth lines. Therefore a BUEHLER IsoMet 1000 Precision Saw (thickness of the diamond saw blade: 0.8 mm)

(Fig. 6 (B)) is used. This side of LSG is grinded with waterproof sandpaper grade 1200 (grain size 15 µm) and following embedded in Araldite.

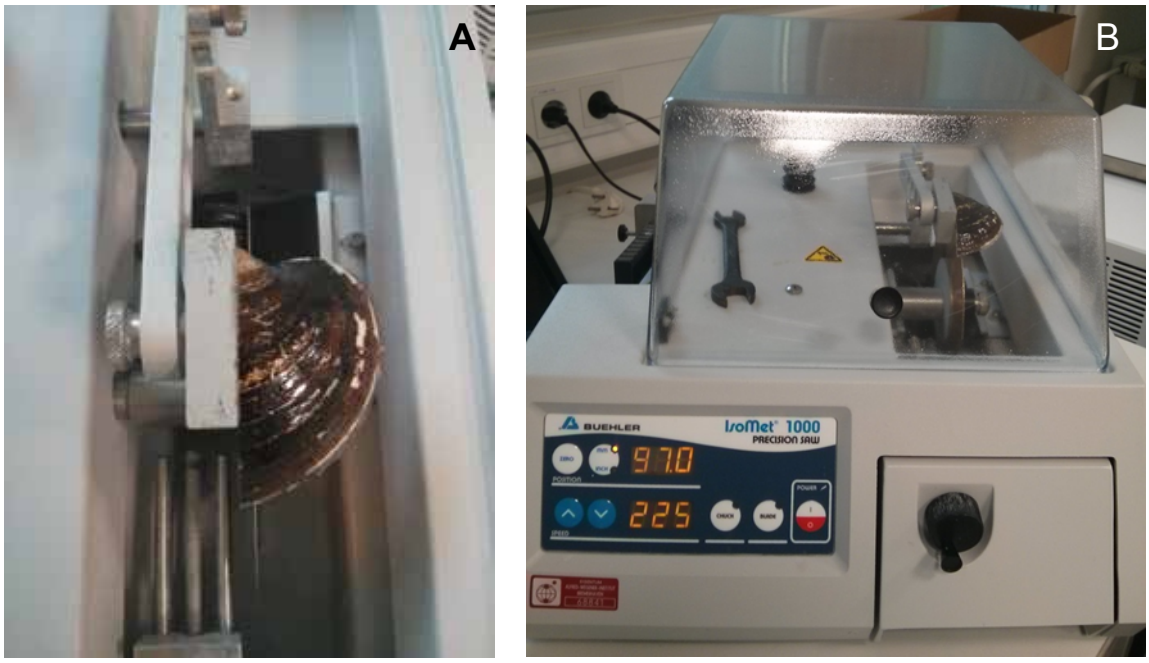


Fig. 6: Low-speed saw used for cutting bivalve shells on the line of strongest growth. (A) One shell of *A. islandica* is glued on a metal block with Crystalbond. (B) Shells were cut with a rotation speed of 225 rpm.

2.4.5 Embedding

Rings of aluminum covered by Teflon are fixed by screws to a plate. Additionally, samples were fixated by superglue to avoid that they tip over. After this pretreatment, Araldite 2020 has been filled into the aluminium rings until bivalve shells have been covered. Like this they have been set to harden in an oven at about 50°C for about 24 hours.

Afterwards, the embedded samples are glued on lapped glass-slides using two-component adhesive EPO-TEK 301 and set to harden for another 24 hours in a special press gadget (Fig. 7 (A)). Bivalves with higher ontogenetic ages were embedded in bigger aluminium rings and they were pressed to the glass-sides on two points (Fig. 7 (B)). EPO-TEK is mixed in relation to three parts of Part A and one part of Part B and the two components are stirred for a few minutes until the liquid remains clear.

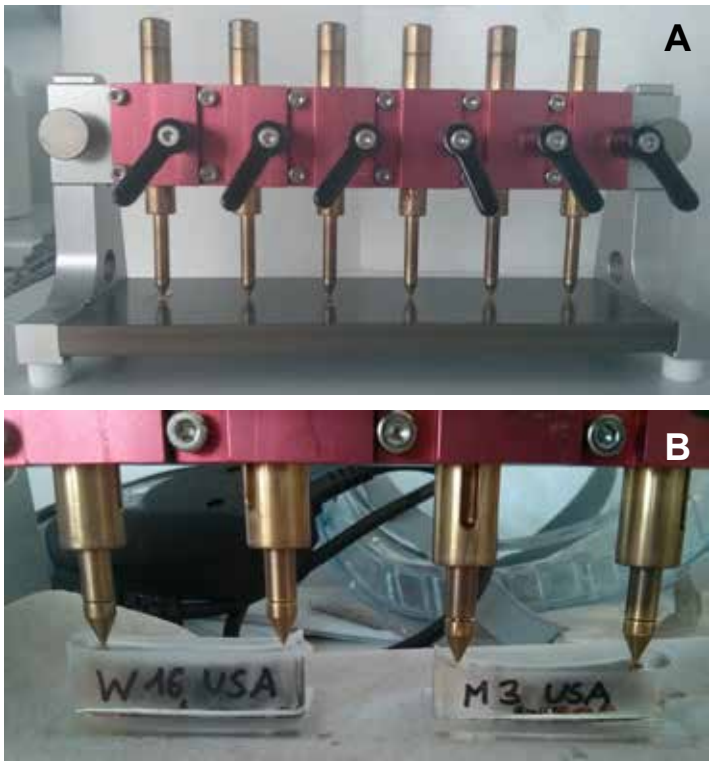


Fig. 7: (A) Special press gadget. (B) Bivalves with higher ontogenetic age are embedded in bigger aluminium rings and they were pressed to the glass-slides on two points.

Preparing thin-sections of bivalve shells, the samples fixed to lapped glass-slides are cut down to a thickness of 200 µm by a low speed saw (procedure as described in Subsection 2.4.4).

2.4.6 Lapping and polishing of shell sections

Following the samples were lapped down to 30 - 60 µm (Fig. 8 (A)). Thereby the glass-slides as well as the samples should have the identical thicknesses, otherwise one or even both samples can be damaged. Firstly, annual growth patterns can be seen in the thin-sections. Since microincrements seem to appear only in a small range of thickness, thin-sections have been checked permanently if microincrements became visual from about 100 µm thickness onwards. On the other hand, if carbonate of the shell starts to polarize under the polarized light microscope thin-sections are too thin and microincrements cannot be identified.

After reaching the wanted thickness of about 30 µm samples were ground by hand with sandpaper grade 2400 and 4000 (grain sizes of 10 µm and 5 µm respectively) to obtain a highly reflective surface (Fig. 8 (B)). Occasionally additional polishing pastes with smaller grain sizes (3 µm, 1 µm or 0.3 µm) were needed.

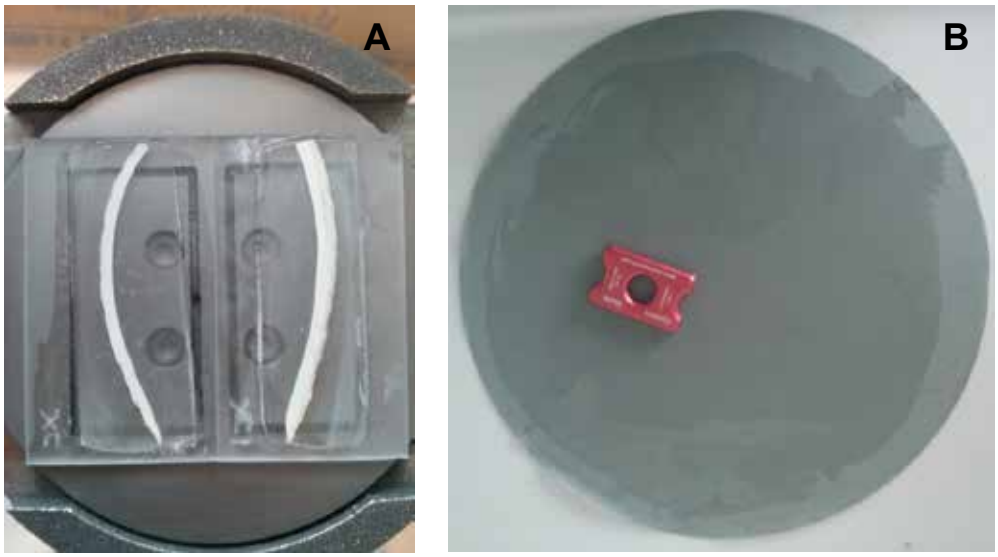


Fig. 8: (A) Bottom side of the supporting head of the lapping and polishing machine. The two thin-sections are cut down to 200 µm and currently in the lapping process. (B) Sandpaper with different grain size is used to grind and polish the thin-sections. For this, glass-slides were mounted into the red holder and moved in irregularly circles over the sandpaper.

2.4.7 Trial and error approaches

No embedding

An approximately 1 mm thick part of the valve is glued on a lapped glass-slide with EPO-TEK 301 with the LSG directly glued on the glass-slide (Fig. 9 (A)). This is done to ensure that the growth pattern later on identified is directly on the LSG rather than 1 mm away. Samples have been lapped until being approximately 35 µm thin. Several problems occurred and have led to the conclusion that this method is not suitable for the preparation of thin-sections. Scratches caused by the saw have to be removed by grinding the shell material before gluing it onto the glass-slides, but this turned out to not be feasible without damaging the bivalve section. Moreover it was not possible to glue the thin-sections of the bivalve shell plan-parallel to the lapped glass-slides. Due to uneven pressure onto the sample and especially towards the ends of the shell (Fig. 9 (B)) by the pressure gadget device (Fig. 7) the preparation of plane-parallel thin-section failed.

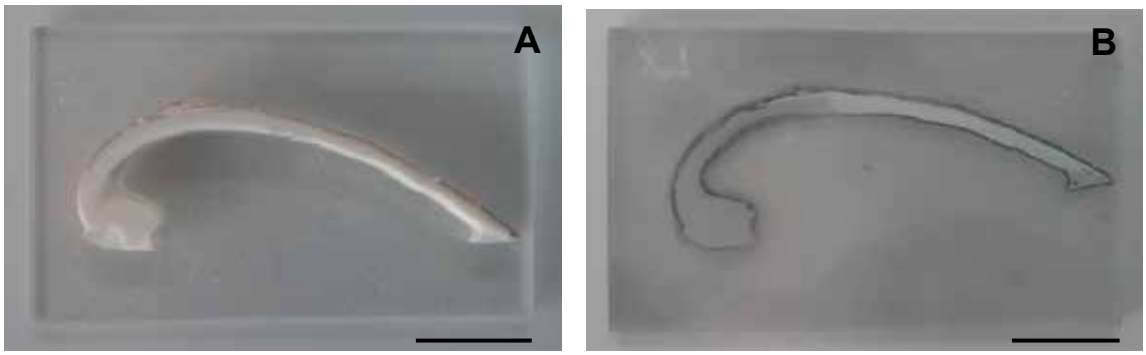


Fig. 9: (A) Cross-section of *A. islandica* is glued on a lapped glass-slide with EPO-TEK. (B) Lapped cross-section of *A. islandica* (shell-ID: 090928). Most parts of the shell material are lost and in the remaining shell material, there are no microincrements visible. The black line in both images illustrates scale of 1 cm.

Abele-System

Another idea to prepare thin-sections was to lap them down to 60 µm with the LOGITECH CL50 Compact 50 Lapping/Polishing Machine and to continue down to a thickness of about 60 µm with the ABELE-System. Even though this method succeeded, it has been considered a failure since it caused big and deep scratches, which could hardly be removed by grinding. Additionally, this method is quite time-consuming and resulting thin-sections are considered less good than lapping approach with the Lapping/Polishing Machine.

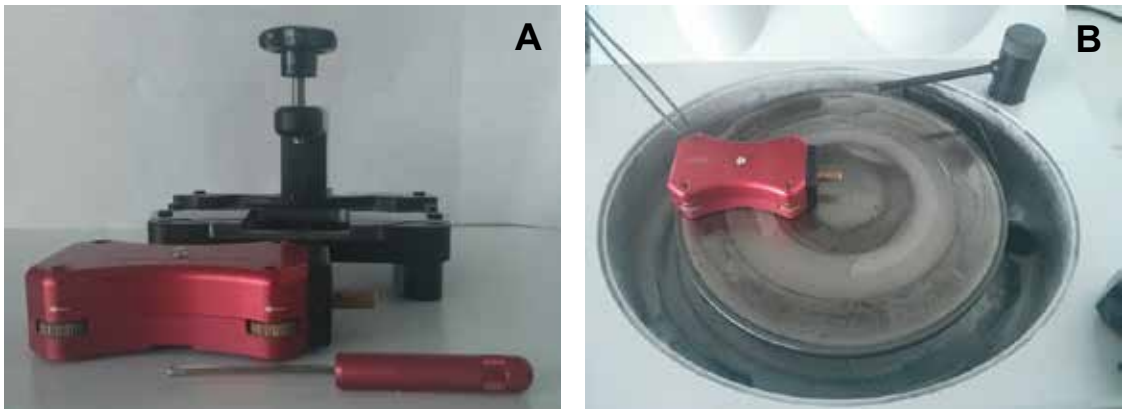


Fig. 10: ABELE-System, which has been used for grinding thin-sections. (A) Complete ABELE system components which are necessary to adjust the thickness. (B) The red glass-slide holder needs to be pressed onto the rotating grinding disc while a constant water flow wets the disc. Two different discs with different grain size 15 µm and 10 µm respectively have been used in this study.

Further observation

Shell material coated with WIKO metal epoxy instead of Araldite 2020 cannot successfully be lapped down to a thickness of 30-60 µm, which would be necessary to visualize microincrements within the outer shell layer. Even if those samples have subsequently been embedded in Araldite, shell material started to break off at a thickness of about 100 µm.

2.5 Additional attempts to improve the visualization of microincrements

Several additional laboratory steps have been tested to increase the visibility in thin-sections. As such it was tested if the microincrements become better visible after cleaning and rinsing in an ultrasonic bath. Two samples were rinsed for 10 min with settings chosen as follows: function: sweep, frequency: 35 kHz, power: 100% and heating off. No difference or improvement could be seen afterwards. Furthermore, different chemicals as well as further visualization techniques were tested and are described in the following.

2.5.1 Etching of thin-section with Mutvei's solution

One thin-section of *A. islandica* (sample-ID: AI-WaHe-25R) was etched in colorless Mutvei's solution (Schöne, 2005a) for 10 minutes at room temperature.

2.5.2 Bleaching of thin-section with hydrogen peroxide (H_2O_2)

An additional attempt to improve the visualization was to bleach one thin-section (sample-ID: AI-WaHe-25R) with hydrogen peroxide (31%) at different time intervals (time intervals increases from 1 min up to 20 min, see Fig. 14).

2.6 Visualization techniques

In this section several applied techniques that have been used to visualize microincrements in bivalve shells will be described.

2.6.1 Transmitted and reflected light microscopy

Magnified images have been produced by using a light microscope. Thereby two lenses, the objective and the ocular, work together and create the final magnification of the object (Murpy and Davidson, 2012). Here, it is necessary to distinguish between transmitted- and reflected light microscopy. Using a transmitted-light microscope the sample, which has to be translucent, is illuminated from below and observed from above. In contrast, using a reflected light-microscope the light is reflected by the sample, which is non-transparent. Samples have been illuminated from one side or directly from above (Murpy and Davidson, 2012).

Analyses of intra-annual growth patterns were conducted on digitized images taken by an Olympus DP 70 camera mounted on a Zeiss Axioskope (software: Olympus DP-Soft). Overview images of freshwater bivalves were taken using x20 and x40 magnifications. Detailed images of freshwater shells used for analyzing microgrowth patterns were taken with a x100 objective and immersion oil. Detailed images of *A. islandica* were taken with magnifications of x10 and x20. Afterwards the images were edited by Adobe Photoshop

CS5.1. The microincrements were counted and measured using the software PANOPEA (© 2004 Peinl & Schöne). Stitched images were put together by Microsoft Research Image Composite Editor (ICE).

2.6.2 Scanning electron microscope (SEM)

Firstly, the sample has been glued on a stub using double-sided adhesive tape. Since the thin-section was bigger than the sub, “CCC-bridges” (dark grey lines in Fig. 11 (B)) from the sub to the border of the glass-slides have to be glued. Edges of the glass-slides have been coated with Lite C (Conductive Carbon Cement). Afterwards, glue has been set to harden for approximately 30 min. The sample was sputtered with gold for 2 minutes (Fig., 11 (A & C)). Afterwards SEM has been run with the following adjustments: cathode 1.78 and beam 10.0 kV.

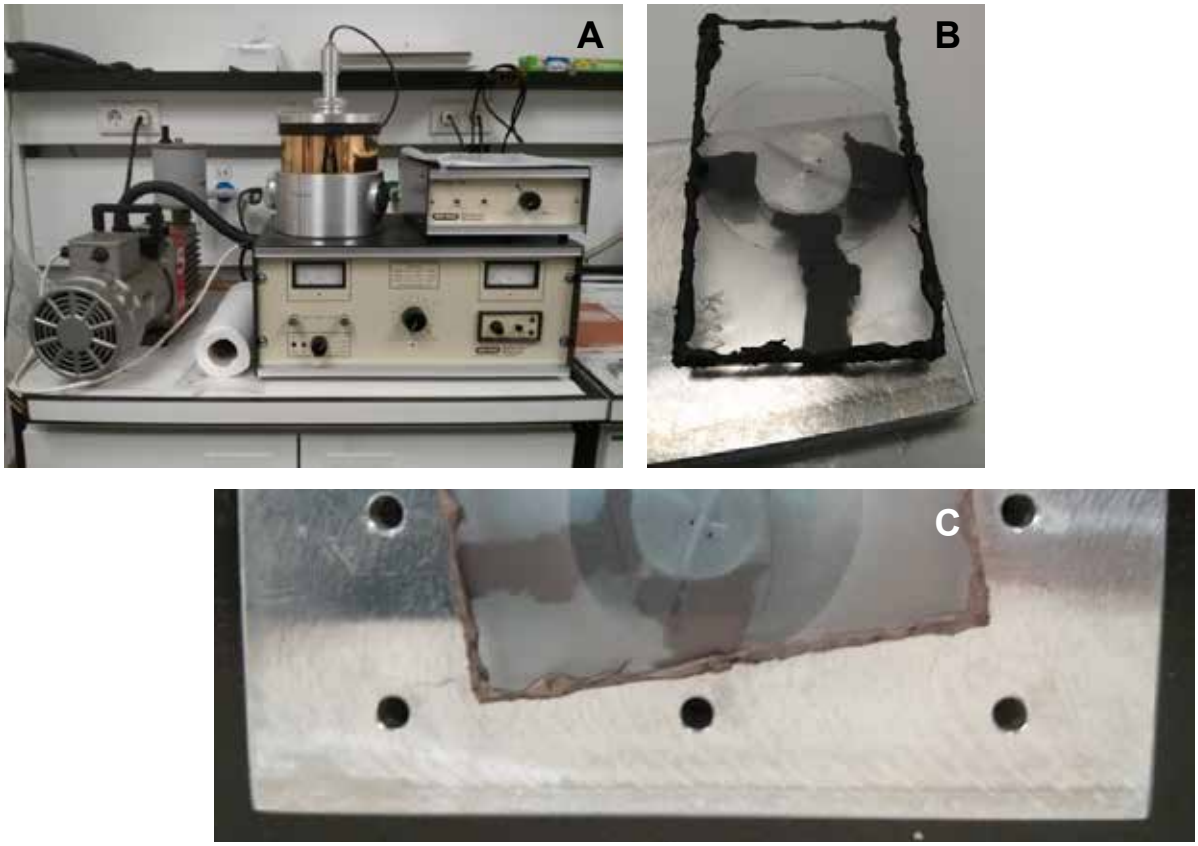


Fig. 11: (A) Sputtering machine. (B) Thin-section of *A. islandica* coated with Lite C. This preparation is necessary before the sample can be sputtered with gold. (C) Gold-sputtered thin-section.

2.7 Thick-section preparation of bivalve shells

Thick-sections of *A. islandica* and *Unio* sp. were prepared to correlate the measured years to calendar years. Moreover they helped to orientate in the thin-section of the same shell. Therefore shells of both genera have been coated twice with Araldite 2020 to prepare 3 mm thick-sections. During cutting of the shell the first cut is done 3 mm right of LSG and the second directly on LSG (Section 2.4.4). The cut thick-section is mounted with metal epoxy resin on glass-slides, the LSG above. Additionally, they have been grinded with sandpaper of grain size 15 µm, 10 µm and 5 µm to get a polished surface.

To visualize annual growth increments the thick-sections were dyed with Mutvei's solution (Schöne et al., 2005b) at 38°C for 23 min. The tree components of Mutvei's solution are acian blue, glutaraldehyde and acetic acid. The solution etches the carbonate, fixes the organic structure and stains simultaneously mucopolysaccharides and glucosamids which are concentrated in the range of annual growth lines (Schöne et al., 2005b). Finally, samples were rinsed several times with aqua deion and air-dried.

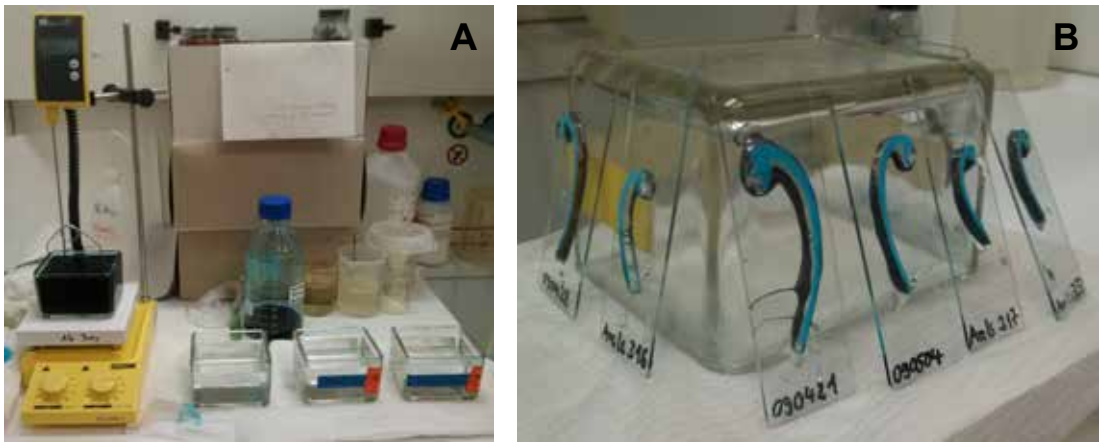


Fig. 12: (A) shows the necessary laboratory equipment and chemicals for dying thick-sections with Mutvei's solution. (B) Thick-sections dyed with Mutvei's solution. They are just air-

2.8 Removal of noise in growth records

The standardized growth index (=SGI) is dimensionless and describes how growth deviates from the average growth trend. A smoothed curve of the growth trend results from the low pass Gaussian digital filter (c.f. Schöne et al., 2003):

$$SGI_{(Gaussian)} = \frac{100 \cdot SGI_{(t)} + 99 \cdot SGI_{(t-1)} + 99 \cdot SGI_{(t+1)} + 95 \cdot SGI_{(t-2)} + 95 \cdot SGI_{(t+2)}}{488}$$

3 Results

3.1 Thin-section preparation

3.1.1 Arctica islandica

Thin-sections were prepared as described in Subsections 2.4.1-2.4.6. Other pathways which were tried (Subsection 2.4.7) have failed. Thin-sections of fourteen *A. islandica* specimens were successfully prepared and have been used to visualize microincrements. Even though all shells were prepared in exactly the same manner, the results differ concerning the visibility of the microincrements in some of the thin-sections. One challenge to visualize microincrements in thin-section of *A. islandica* is the so called “white band”. A white area in the outer layer of shell which is parallel to the periostracum complicates or even prevents the measurement of microincrements.

3.1.2 Unio *sp.*

Two thin-sections of freshwater bivalves of *Unio* sp. were prepared exactly the same pathway as shells of *A. islandica* (c.f. Subsection 3.1.1). Microincrements of *Unio* sp. are smaller than those of the ocean quahog *A. islandica*.

3.2 Additional attempts to improve the visualization of microincrements

3.2.1 Etching of thin-sections with Mutvei’s solution

One thin-section of *A. islandica* (shell-ID: AI-WaHe-25R) was etched in colorless Mutvei’s solution. Before etching microincrements could be seen under the microscope (Fig. 13 (A)). After etching the whole shell appears darker, but there was no improvement in the visibility of microincrements. Single microincrements could not be discerned afterwards (Fig. 13 (B)).

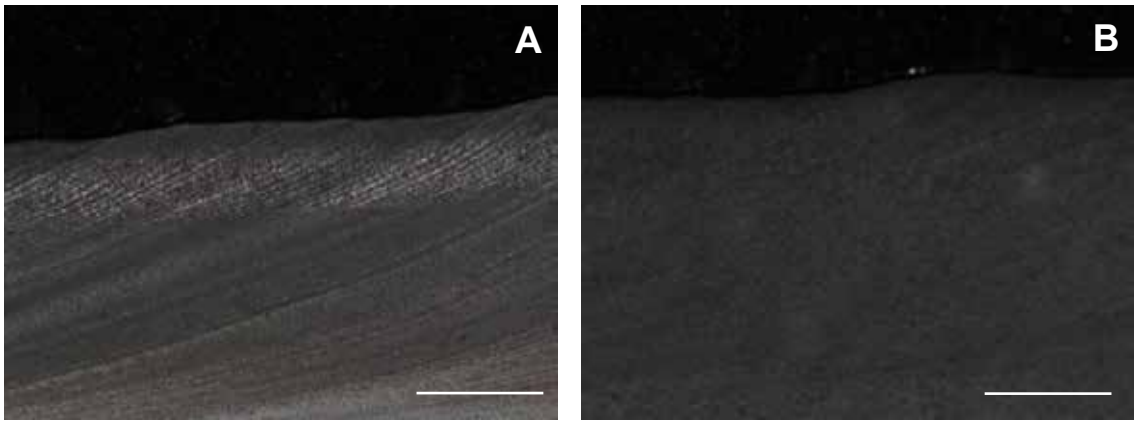


Fig. 13: Before (A) and after (B) images of one thin-section of *A. islandica* (shell-ID: AI-WaHe-25R). (A) Microincrements can be seen. (B) After etching with Mutvei’s solution for 10 min microincrements are no longer visible. These images are taken with a x10 magnification. The white line in both images illustrates scale of 200 µm.

3.2.2 Bleaching of thin-sections with hydrogen peroxide (H_2O_2)

Before bleaching the thin-section, the microincrements were visible. As seen in Fig. 14, the shell material is getting brighter, but visibility of microincrements is not increased. After an incubation-time of 30 min the microincrements are hardly visible (Fig. 14 (F)). The microincrements look blurry and single ones cannot be discerned any longer.

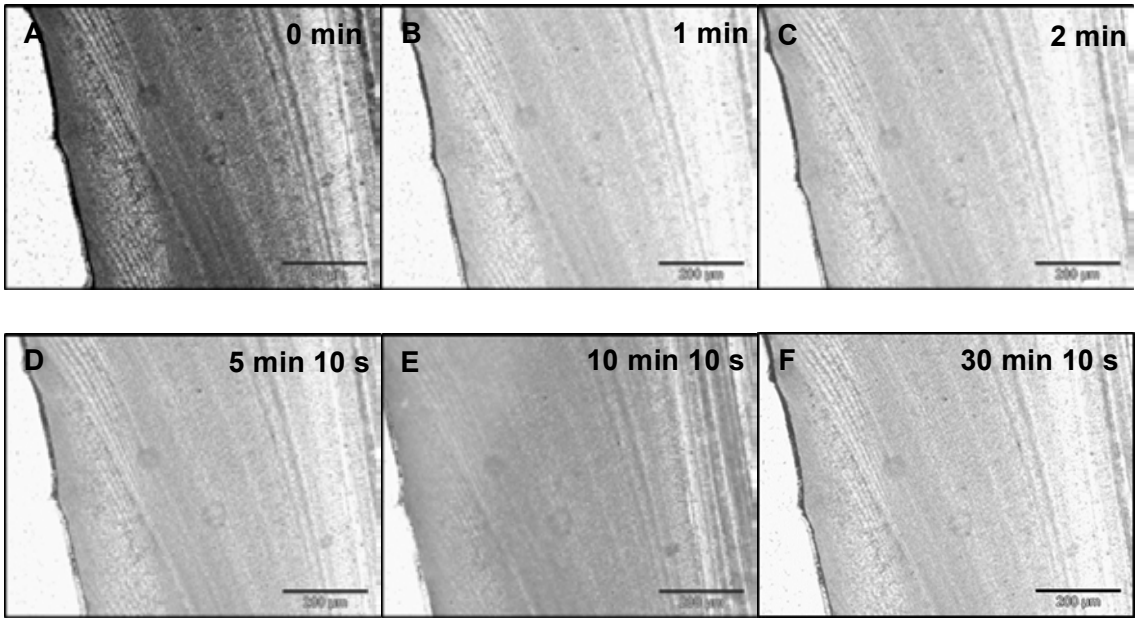


Fig. 14: Illustration of an *A. islandica* thin-section (shell-ID: AI-WaHe-25R), which was been bleached for up to 30 min. The images show the microincrements before bleaching (0 min; A) and the changes over time (B-F).

3.3 Visualization techniques

3.3.1 Transmitted and reflected light microscopy

Microincrements in thin-section of *A. islandica* and *Unio* sp. are visible using a transmitted-light microscope for visualization (Fig. 15). Reflected-light microscopy on thin-section is not suitable for visualizing microincrements. This is used for non-transparent thick-section samples.

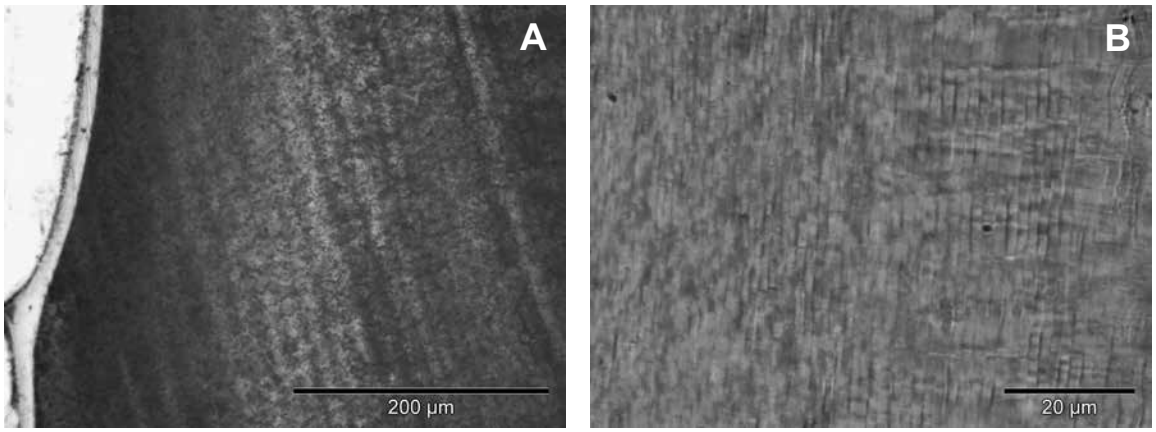


Fig. 15: (previous page) Microincrements are visible using a transmitted-light microscope. (A) shows microincrements of *A. islandica* (shell-ID: 090421) and (B) of *Unio* sp. (shell-ID: LB-LaMa-02R). They look different and microincrements of *Unio* sp. (x100 magnification) are significantly smaller than those of *A. islandica* (x20 magnification). In both images the periostracum is on the left side.

3.3.2 Scanning electron microscope (SEM)

Neither annual growth patterns nor daily microincrements were visible on images taken by SEM. Deep scratches can be seen on the surface (Fig. 16).

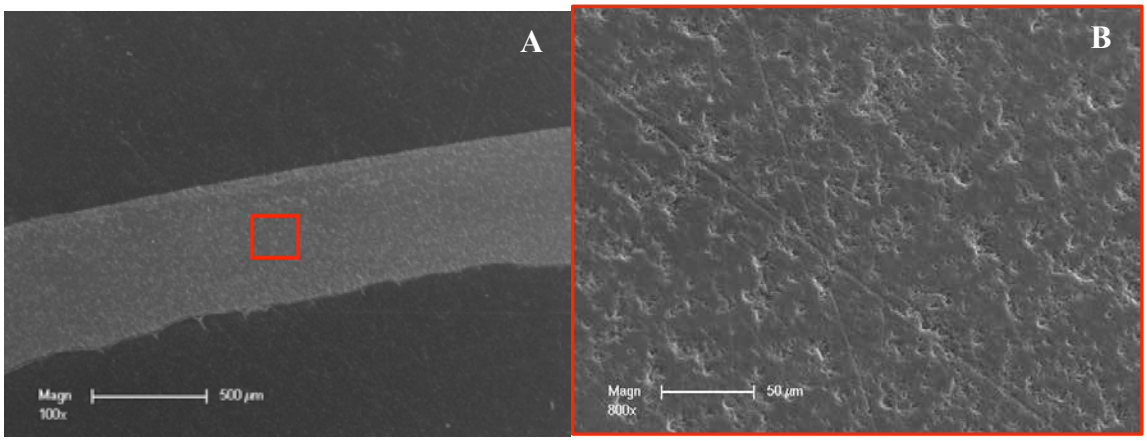


Fig. 16: (A) Overview SEM image of AI-WaHe-40. (B) Detailed image as indicated by red box in A. Deep scratches are seen on this image. Neither annual growth structures nor daily ones are visible.

3.4 Measurements

3.4.1 Arctica islandica

Measurements of two thin-sections of *A. islandica* were conducted. It was not possible to measure and count the microincrements throughout an entire ontogenetic year. All three measurements of one part of ontogenetic year 7 done in shell Ars Is 317 (Iceland) (Fig. 17), which have been done to minimize the error in measurement, show a similar trend. Firstly, the overall trend of microincrement widths increases and then decreases again. Going in detail, the single measured microincrement widths permanently increase and decrease. The growth trend is also illustrated in a further figure (Appendix 1) where measurements have been filtered using a Gaussian filter.

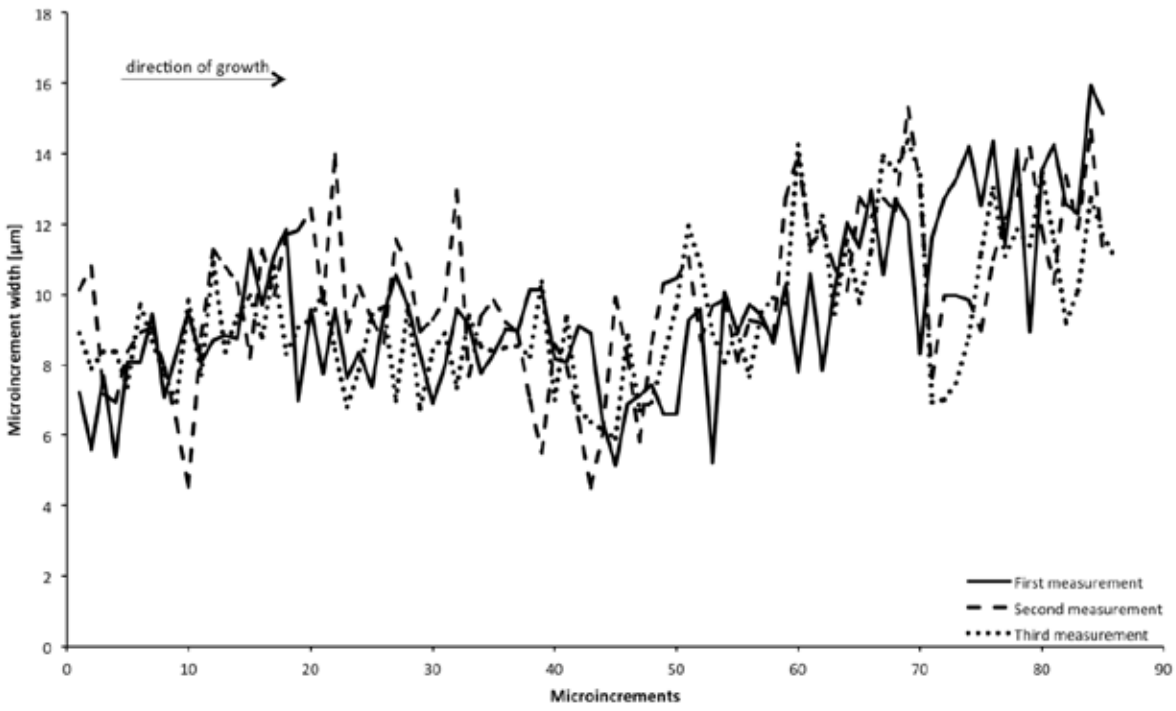
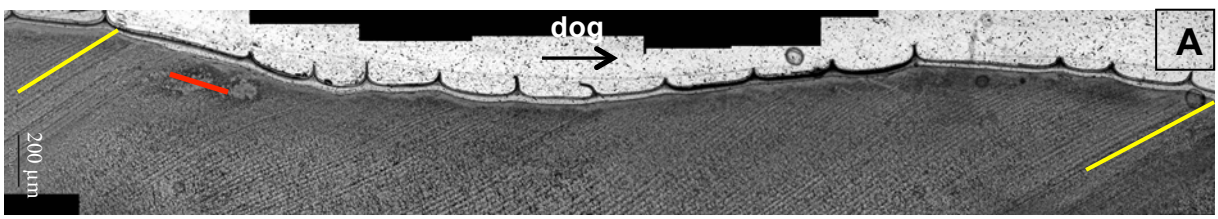


Fig. 17: Three measurements of one section of the shell of *A. islandica* (sample-ID: Arc Is 317). The shell was sampled alive in Iceland in 2008. Individual microincrements are counted and measured. A trend of increasing microincrement widths starting approximately from microincrement 45 is observed.

In Fig. 18 (B) differences in the second part of the measurements between the first and the second measurement of the microincrement widths are seen. Due to this difference the measurements have been checked for any errors in measurement. During the first measurement of the second part an area consisting 12 microincrements was measured twice. Therefore the twice measured microincrement numbers 61-70 of the first measurement were deleted. Fig. 18 (C) shows the edited growth trend. Thereby, both growth curves (second part of measurements) follow a more equal trend than before. Due to single microincrements which could not be discerned (red bar in Fig. 18 (A)), it was not possible to analyze this part of the shell consecutively. The first part (until microincrement number ~19, see red bar in Fig. 18) was measured until the microincrements were not visible and measurements go on after they could be seen again.



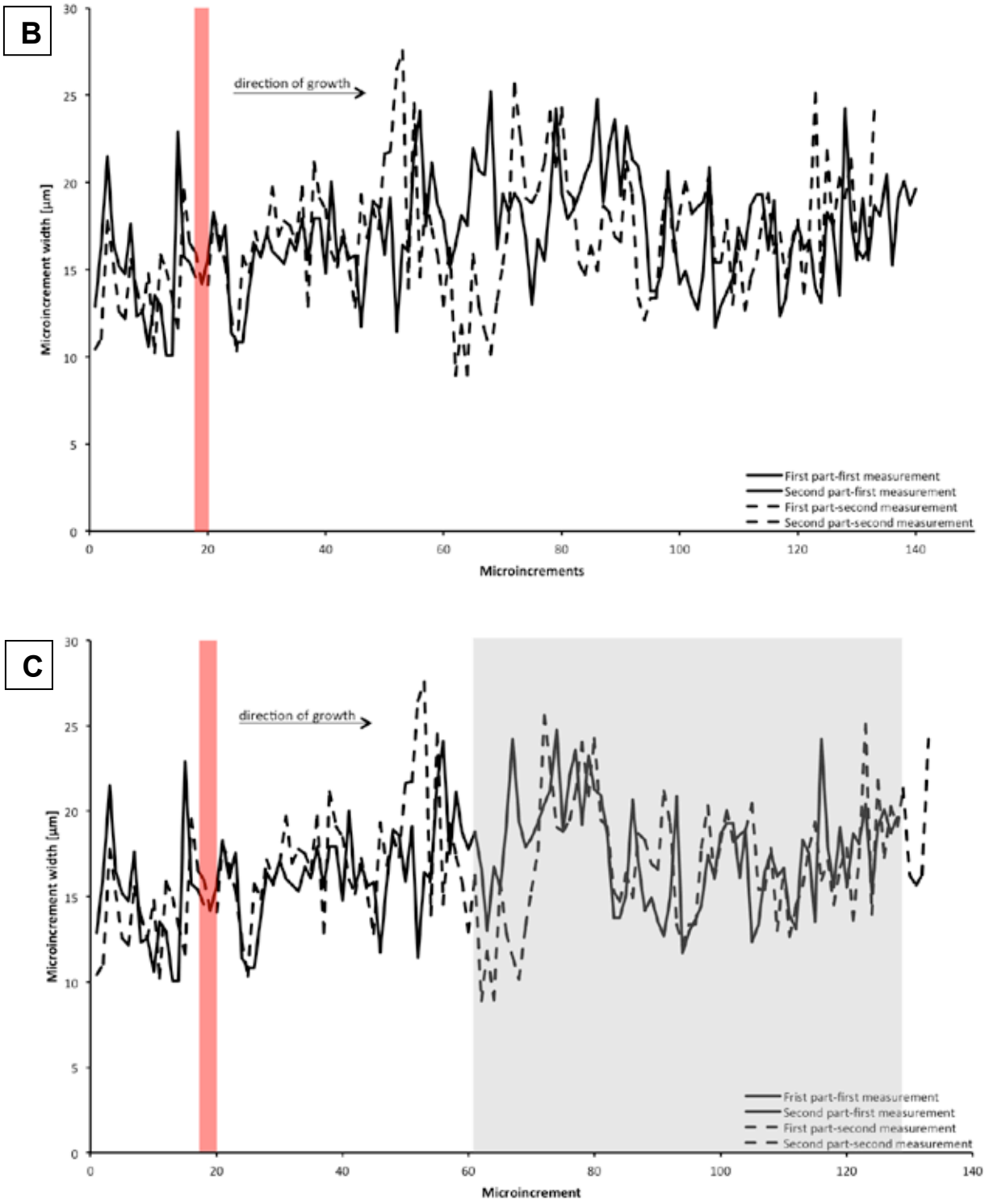


Fig. 18: Illustration of microincrement widths of one ocean quahog *A. islandica* (sample-ID: W 16), which was collected in the USA. The red bar (A, B & C) illustrates a break during the measurements. (A) shows the images which are the basis of these measurements stiched together. The yellow bars in (A) illustrate the start and stop of the measurements. The measurements were done in direction of growth (dog). The scale of this picture is the black bar in the left corner and is 200 µm. Consecutive measuring had to interrupt due to blurry appearance of microincrements. The first part includes 18 microincrements for the first measurement and respectively 19 microincrements for the second measurement. The grey accentuation in (C) illustrates the area which differs from (B) due to editing.

3.4.2 Unio *sp.*

Measurements in freshwater bivalve (shell-ID: LB-LaMa-02R) have been done. This shell is characterized by a break (see Fig. 19 (A)).

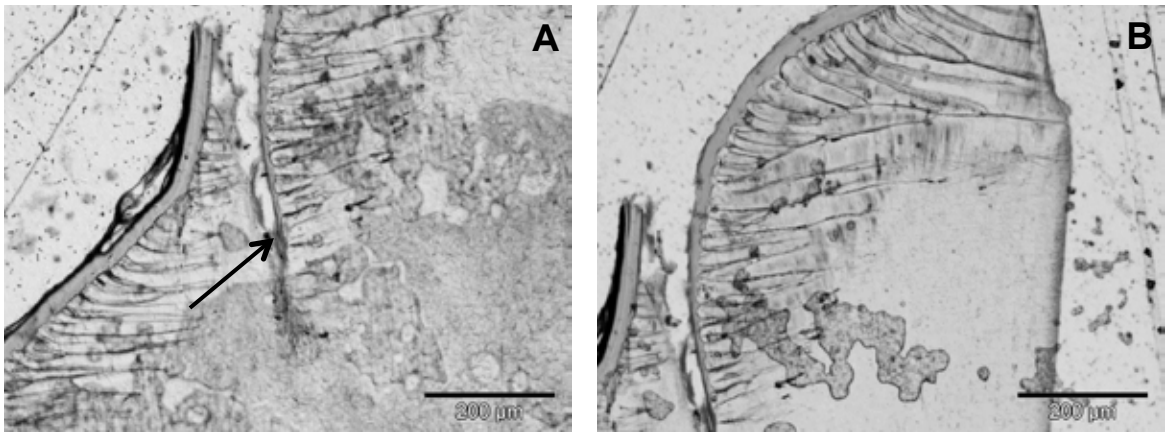


Fig. 19: Images of a thin-section of *Unio* sp. (shell-ID: LB-LaMa-02R) taken under the Axioscope at x10 magnification. In (A) the break is shown (arrow). It is illustrated as red bars in Fig. 19. Due to the crack the microincrements are not measured consistently. (B) Illustrates the part of the shell where the "measurements after crack" were done.

Measured microincrement widths show a growth trend. In Fig. 20 the microincrements firstly stay relatively constant until microincrement number 100, then decrease and reaching the minimum value of microincrements measured in this shell at microincrement number ~135. Then the microincrement widths of approximately 100 microincrements increase. After reaching a peak at microincrement number ~230, the widths of microincrements decrease again until microincrement number 321. This weak trend in microincrement widths patterns in *Unio* sp. covers a range of approximately 200 microincrements.

After the break the average value of microincrement widths increase. These aberrations can also be caused by different distance to the periostracum because microincrements are getting smaller going from the outer shell layer to the inner shell layer.

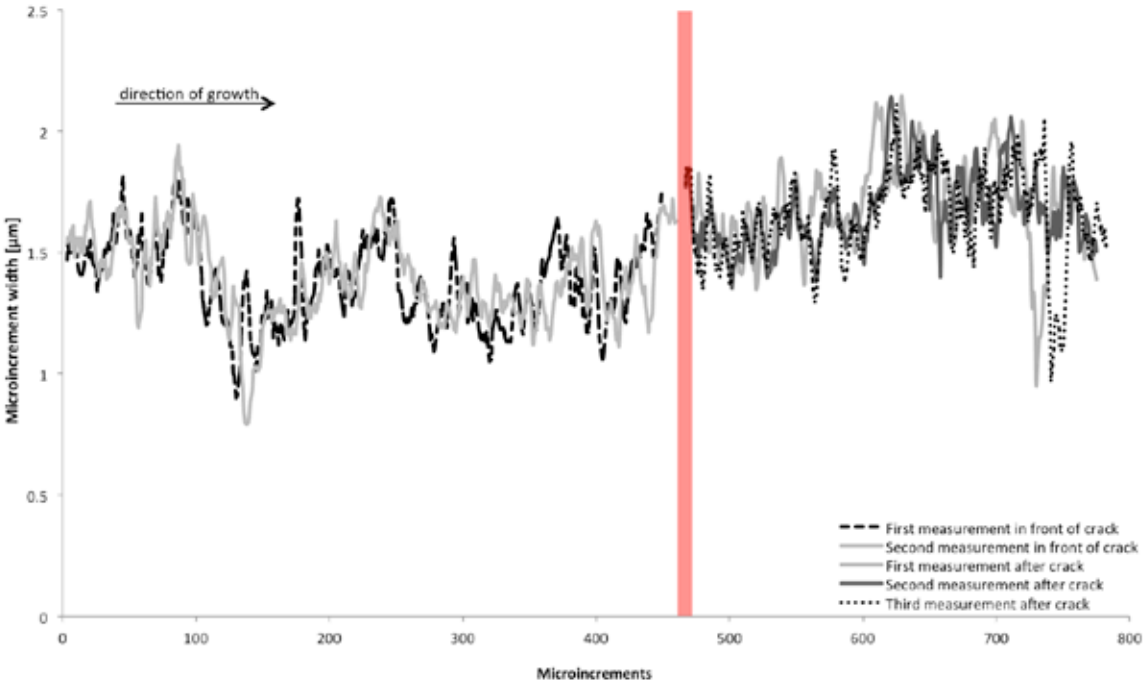


Fig. 20: Illustration of the microincrement width in one freshwater shell (sample-ID: LB-LaMa-02R). The red bar illustrates a break which interrupts consistent measurements. The microincrements were measured until the ventral margin. Measurements presented here have been filtered using a Gaussian filter. An additional figure showing the raw data can be found in Appendix 3.

Moreover the microincrement widths have been measured in another shell of *Unio* sp. (shell-ID: LB-LaMa-03R). The second measurement differs strongly from the other two measurements. Especially the peak for microincrement number 84 of the second measurement stands out. This is the reason why in Fig. 21 (B) the second measurement has been deleted. Both lines (first and third measurement) show a similar growth trend. Starting at around measured microincrement 175, the microincrement widths firstly increase until microincrement number 240, stay constantly until microincrement number 320 and afterwards the microincrement widths decrease. Those described measurements cover a range of approximately 200 microincrements. Before microincrement number 175 the microincrement widths decrease slowly.

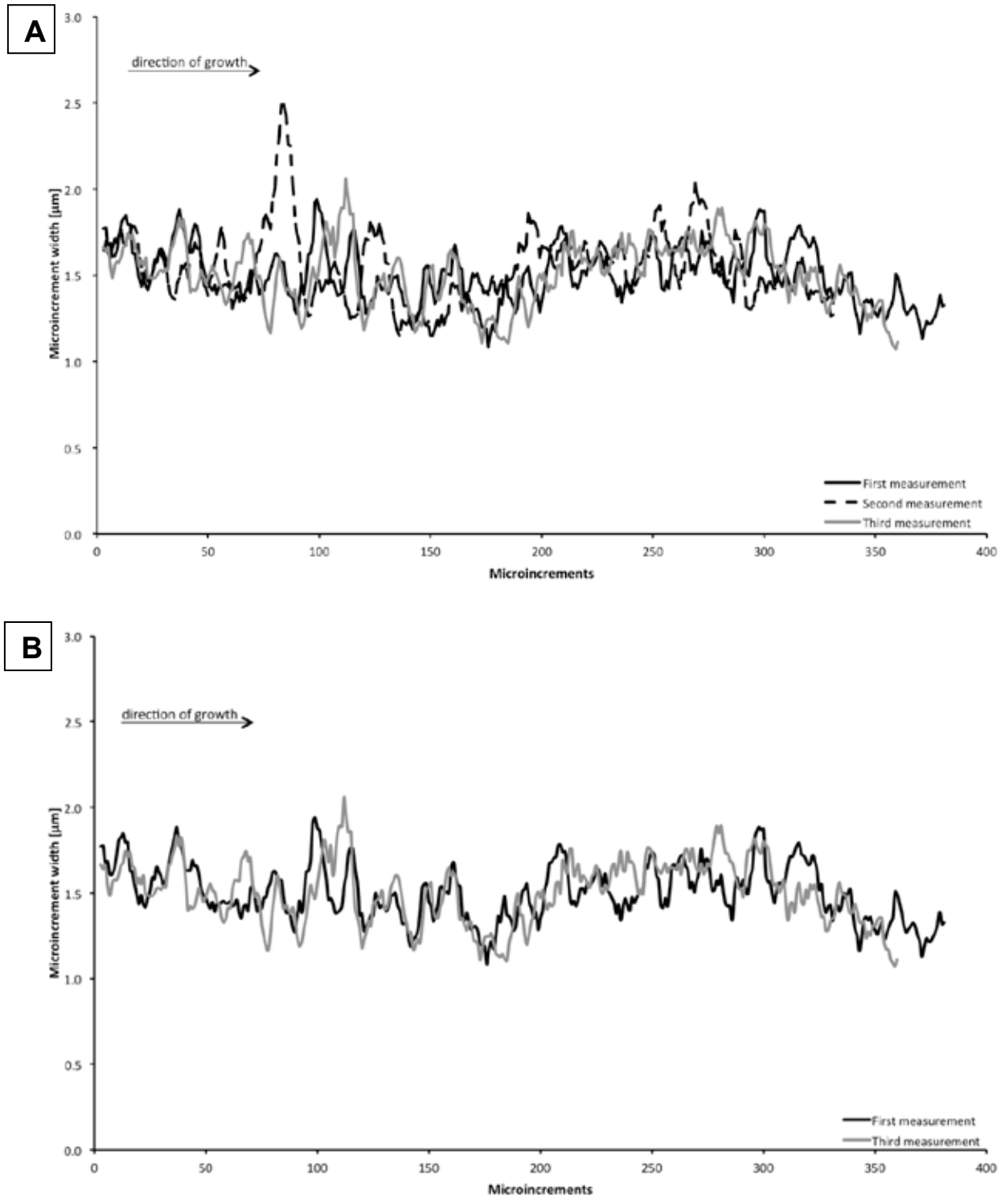


Fig. 21: Illustration of the growth trend of one *Unio* sp. (shell-ID: LB-LaMa-03R). The measurements which are the basis of these figure are filtered by the Gaussian filter. (A) shows the measurement of the width of microincrements of all three measurements which were done. (B) illustrates only the results of the first and third measurement. The second measurement differs extremely from the other two.

The boxplot in Fig. 22 shows the variance of microincrement widths in *Unio* sp. The dots illustrate outliers, respectively the maximum and minimum. The box covers the range in which are 50% of the mean values. The beginning of the box illustrates the lower quartile and the stop the upper one. The bar in the box is the median. This value is 1.5 µm for the shell material collected in the Lago Maggiore and about 0.25 µm higher, around 1.75 µm for the *Unio* sp. collected in Germany (Beierlein, 2011).

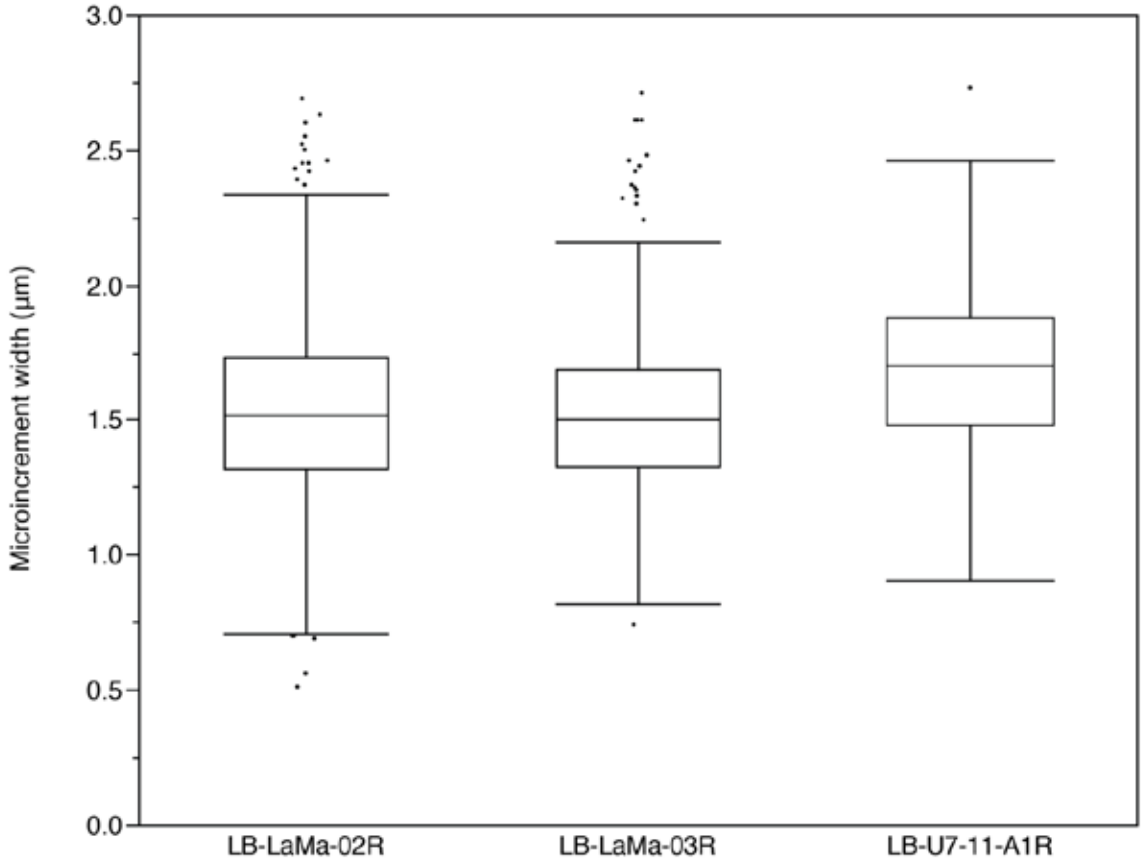


Fig. 22: Graphical illustration of the variance of microincrement width in the freshwater mussels *Unio* sp. Thin-sections of shells LB-LaMa-02R and LB-LaMa-03R were prepared by me. Data for LB-U7-11-A1R were taken from Beierlein, 2011. Shell material LB-LaMa-02R and LB-LaMa-03R were sampled in Lago Maggiore, LB-U7-11-A1R in the Unstrut River near Wallhausen.

The maximum for LB-LaMa-02R is 2.69 µm and respectively the minimum 0.51 µm. The measured width of microincrements in shell LB-LaMa-03R ranges between the maximum value of 2.71 µm and the minimum one of 0.74 µm. For shell LB-U7-11-A1R a minimal value of 0.91 µm is measured and respectively a maximum one of 2.73 µm. Generally, the range of measured microincrement widths is between 0.5 and 2.5 µm.

3.4.3 Comparison between A. islandica *and* Unio *sp.*

Comparing the measured microincrement widths of both genera, those values differ significantly (see Fig. 23). The maximum microincrement width measured in *A. islandica* is 27.56 µm and respectively 2.73 µm for *Unio* sp. The minimum value measured for *A. islandica* is 4.50 µm and respectively 0.51 µm for *Unio* sp. The boxplot in Fig. 23 of *A. islandica* is based on a smaller dataset (3333 measured microincrements) than those of *Unio* sp. (529 measured microincrements).

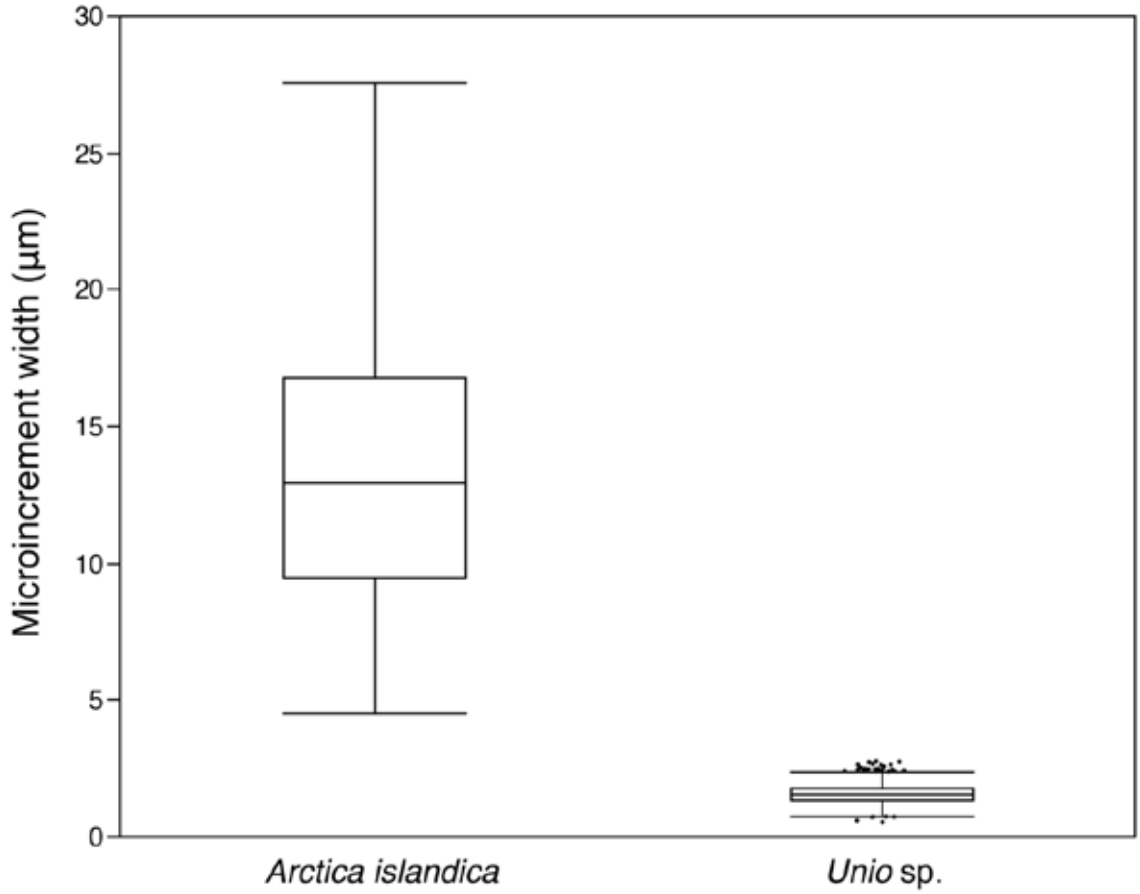


Fig. 23: Graphical comparison of variance of microincrement widths between *A. islandica* and *Unio* sp. This figure is based on all measured microincrement widths for *A. islandica* (shell-IDs: Arc Is 317 and W 16) and respectively for *Unio* sp. (shell-IDs: LB-LaMa-02R, LB-LaMa-03R and LB-U7-11-A1R).

4 Discussion

4.1 Thin-section preparation

Several steps are necessary to prepare thin-sections successfully: coating, cutting, embedding, gluing on lapped glass-slides, cutting down to 200 µm and finally, lapping and polishing. The most challenging part is the last one. Thin-sections have to be lapped down to a thickness of approximately 30 µm. Even the supporting head of the Lapping/ Polishing machine is correctly adjusted and theoretically, the sample should not get thinner, shell material can be lost very fast. Especially the ends of the shell often break off.

4.1.1 Arctica islandica

Microincrements in shells of *A. islandica* are visible using a transmitted-light microscope, but they look blurry with increasing magnification. In none of the prepared thin-sections of *A. islandica* microincrements could be seen from one winter line to the next. It is challenging to discern between single microincrements and to measure the width of those due to “white band” (Subsection 3.1.1). This part of the shell appears white under a transmitted-light microscope and microincrements are hardly visible.

It is presumed that the “white band” is associated with shell structure. The “white band” is parallel to the periostracum in the outer layer of the shell and complicates measuring of microincrements because those are hardly visible in that part of the shell. Maybe the organic content differs from the one at other shell parts. Working with transmitted-light the white color point out that the shell structure is less dense than the parts of the shell appearing darker. Moreover Araldite “penetrates” in the shell during coating. The “white band” is not visible in thin-sections of the freshwater bivalve. But *Unio* sp. has a different shell structure, which could explain why Araldite do not “penetrate” in those shells. Furthermore the white band could be caused during lapping. If this can explain the white area parallel to the periostracum of one shell, it would mean that this part of the shell is more stressed during the lapping process than the rest of the shell.

At the beginning of my research, I have done several thin-sections of one shell. There are big differences in the “quality” of thin-sections even all were prepared the same way. In all thin-sections prepared from one shell (e.g. AI-WaHe-25R) microincrements are well or bad visible. Therefore, in further studies more thin-sections have to be prepared than actually needed. Those differences exist between and within populations. Intraspecific competition explains different visibility of microincrements within one population.

4.1.2 Unio *sp.*

Using a transmitted-light microscope, microincrements are visible in thin-sections of freshwater bivalve *Unio* sp. Due to different shell structure and biomineralization process the microincrements of freshwater bivalves look completely different than those of *A. islandica*. Microincrements of *Unio* sp. are significantly smaller than those of the ocean quahog *A. islandica* (Fig. 23). A high magnification of x100 and immersion oil is necessary to visualize, count and measured microincrements in thin-sections of *Unio* sp. Moreover those thin-sections have to be perfectly lapped and polished. Any scratches degrade the visibility of microincrements in freshwater bivalves.

4.2 Additional attempts to improve the visualization of microincrements

4.2.1 Etching of thin-section with Mutvei's solution

The idea of etching one thin-section was to accent the microincrements. For this trial colorless Mutvei's solution was used. Commonly, thick-sections were colored by blue Mutvei's solution (Subsection 2.7). Mutvei's solution accents the topographic relief of growth patterns in thick-sections. This method improves the visualization of growth lines using reflected-light microscopy. But etching thin-sections does not improve the visualization of microincrements (Fig. 13). Those are not visible in etched thin-sections. The visibility of microincrements in thin-sections increases the smoother the surface is. Mutvei's solution dissolves calcium carbonate and destroys the polished surface which is necessary for visualization microincrements. Summing up, etching does not improve the visibility of microincrements in bivalve shells.

4.2.2 Bleaching of thin-section with hydrogen peroxide (H_2O_2)

The idea of bleaching one sample was to improve the visualization of microincrements. In contrast to etching, bleaching should accent the inter-crystalline organic matrix and bright up the grey shadow on the shell. Some samples in total or even parts of them are very dark which complicates the measurement of microincrements. Due to bleaching the shell brightens up, but it does not improve the visibility of microincrements (Fig. 14). Hydrogen peroxide reacts with calcium carbonate of the bivalve shell. The shining surface is destroyed, but microincrements are only visible in thin-sections with perfectly polished surfaces.

4.3 Visualization techniques

4.3.1 Transmitted and reflected light microscopy

Transmitted-light microscopy is used to visualize growth patterns on a daily scale. Microincrements are visible in thin-sections of *A. islandica* and *Unio* sp. being 30-40 µm thick (Fig. 15). Thin-sections thicker than 40 µm are too dark for the visualization of microincrements because to less light shines through the sample. Thin-sections having a thickness of 25 µm are too thin. Those start to polarize and microincrements are not visible. Thin-sections thicker and thinner than the range of 30-40 µm are not suited for the visualization of microincrements.

4.3.2 Scanning electron microscope (SEM)

No growth structures in the shell can be seen. The thin-sections were polished (Subsection 2.4.6) to get a shining surface and removing scratches caused during cutting and lapping, but deep scratches are visible on the SEM images (Fig. 16). Growth patterns are not visible on the images taken by the SEM. Moreover scratches are visible even the thin-sections were polished. The surface of this thin-section was polished, but scanning electron microscopy works with reflected light. As described in Subsection 4.2 transmitted-light has to be used to visualize microincrements in polished thin-sections. Therefore I suggest using etched thin-sections to visualize growth patterns with SEM in further studies.

4.4 Measurements

4.4.1 Arctica islandica

As described before (Subsection 3.1.1), visualizing of microincrements in marine bivalve shell *A. islandica* is quite challenging. To get first information about the widths of microincrements in this species, 90 (Arc Is 317) and respectively 130 (W 16) microincrements were measured in two specimens, from Iceland (shell-ID: Arc Is 317) and the USA (shell-ID: W 16).

Microincrements in *A. islandica* look blurry with increasing magnification. Therefore magnifications of x10 (for Arc Is 317) and x20 (for W 16) were used to take the pictures on which the measurements are based. As seen in Fig. 17 the minimum microincrements widths measured in shell Arc Is 317 is 4.50 µm and respectively the maximum width is 15.94 µm for the ocean quahog collected in Iceland. The minimum value which was measured in another shell of *A. islandica* (shell-ID: W16) is 8.90 µm and the maximum value is 27.56 µm (Fig. 18). Schöne et al. (2005a) specifies the measured width of

microincrements in Dogger Bank (North Sea) with 6-58 µm and in the German Bight (North Sea) with 23-51 µm. This data are based on the fourth year of growth.

The minimum and especially the maximum values measured differ from those in Dogger Bank and German Bight. The results shown in Figs. 17 & 18 do not cover a whole year and the microincrements were measured in different ontogenetic years. Approximately 90 (Arc Is 317) and respectively 130 (W 16) microincrements (for W 16: not consecutively) were analyzed. In contrast, on average 232 microincrements were measured in growth year four (Schöne et al., 2005a). Hence the measured section covers only a quarter (Arc Is 317) and respectively the half of one year (W 16). Besides, it is not known if there is any growth break within the measured microincrements of those shells (Arc Is 317 and W 16). It is known that the microincrement width varies within one year (Schöne et al. 2005a). Moreover it has to be recognized that they are from different localities with different environmental conditions. But no conclusion concerning environment and locality could be done due to missing information and the fact that it was not possible to measure one year consecutively.

Figure 18 (B & C) shows the challenges measuring microincrements in marine bivalve shells of *A. islandica*. As described before (Subsection 3.4.1) Fig. 18 (C) shows the edited dataset. The grey accentuation illustrates the changes in the growth trend based on the first measurement. This error in measurement clarifies the challenges of analyzing microincrements in marine bivalve shells.

4.4.2 Unio *sp.*

A similar growth trend described for shell LB-LaMa-02R (Subsection 3.4.2, Fig. 20) can also be seen in Fig. 21 illustrating the variance of microincrement widths in another shell of *Unio* sp. (LB-LaMa-03R). In Fig. 21 this growth pattern is visible beginning at microincrement number 175. Possibly, this point marks a (winter) annual growth line, but those are hardly visible in thin-sections of *Unio* sp. The following curve covers a range of approximately 200 microincrements as described in Beierlein (2011).

The boxplot (Fig. 22) shows that the median for both freshwater bivalves sampled in the Lago Maggiore (shell-ID's: LB-LaMa-02R and LB-LaMa-03R) and the *Unio* sp. collected in Central Germany (shell-ID: LB-U7-11-A1R) is almost identical. The calculated medians fit together quite well. Shells LB-LaMa-02R and LB-LaMa-03R have lived in the Lago Maggiore in Italy, whereas *Unio* sp. LB-U7-11-A1R has been collected in a river in Central Germany. The locality in Germany is further north than the Lago Maggiore in Italy. Moreover different ontogenetic years were measured in all shells. These reasons explain the insignificant differences in the calculated value of the median. Outliers above the

upper quartile can be explained by errors in measurement. Two microincrements could not be discerned and be measured as one.

4.4.3 Comparison between A. islandica *and* Unio *sp.*

In contrast to *A. islandica* it is easier to visualize microincrements in shells of the freshwater bivalve *Unio* sp. even the microincrements in *Unio* sp. are significantly smaller than those in *A. islandica* (Fig. 23). The average microincrement width for *A. islandica* is 12.5 µm and 1.5 µm for *Unio* sp. Differences in biomineralization processes between bivalve species are crucial if microincrements can be visualized.

5 Conclusions

In this study I focused on the preparation of thin-sections of bivalve shells and furthermore I was interested in examining the potential of such thin-sections as a window to environmental reconstruction on a daily scale. The main focus was on the marine species *A. islandica* and the freshwater bivalve *Unio* sp. and finally, for both species, thin-sections have successfully been prepared. However, during my thesis it was not possible to correlate any environmental data to the data retrieved from the thin-sections. Main challenge in *A. islandica* was the visualization of microincrements itself, whereas in *Unio* sp. it was not possible to correlate environmental data due to missing information about the date of death.

In the following the key findings of my work are summarized:

- Several techniques and methods (Subsections 2.5 & 2.6) have been tested for their potential to visualize microincrements in bivalve shell. Finally, the procedure described in Subsections 2.4.1-2.4.6 is considered the most promising for the preparation of thin-sections.
- Microincrements can be visualized by thin-sections and they are visible in both target species (Subsection 3.4). Microincrements in *Unio* sp. are significantly smaller (1.5 µm on average) than in *A. islandica* (12.*5* µm on average) (Fig. 23).
- In *A. islandica* microincrements became visible using a microscope with transmitted-light and a x10 magnification (Fig. 15). With higher magnification microincrements looked blurry and it was not possible to measure consecutive microincrements over an entire ontogenetic year. However, to get a first impression about the variability in microincrement widths in *A. islandica* measurements were carried out in different shell areas and different ontogenetic years (Subsections 3.3.1 and 3.4.1, Figs. 17 and 18).
- Microincrements in *Unio* sp. have successfully been measured consecutively. Here, a high magnification of x100 and immersion oil were necessary to visualize, count and measure microincrements (Subsections 3.3.1 and 3.4.2, Figs. 20 and 21).
- A weak annual trend in the microincrement width pattern in *Unio* sp. has been found. However, due to missing information on the date of death it was not possible to correlate the measurements to environmental datasets (Subsections 3.4.2 and 4.4.2, Figs. 20 and 21).

6 Outlook

Even though the knowledge on how to successfully prepare thin-sections of bivalve shells has been gained in this study (Subsections 2.4.1-2.4.6) further research on additional shell material is needed in order to verify these results. Due to its exceptional importance as a bioarchive, future work should focus on the visualization of microincrements in *A. islandica*. In the following some suggestions on how to improve the visibility of microincrements are given:

- The abrasive used in this study has a grain size of 9 µm and is grey-colored. Especially in thin-sections of *A. islandica* this led to a dark discoloration under the microscope. It cannot be excluded that the abrasive "penetrated" the shell carbonate. I suggest usage of a white-colored abrasive with an even smaller grain size to simultaneously minimize scratches during the lapping process.
- To improve the visualization of microincrements I suggest a scan using a confocal Raman microscope. Raman maps have a high spatial resolution and can provide information on growth patterns in biological hard parts where conventional methods fail.
- To proof if the microincrement widths differ significantly between localities further studies have to be done. Therefore, it would be essential to measure the microincrements in identical ontogenetic years of shells from different localities.
- Shells of *Unio* sp. seem to be a suitable recorder of the past environment on a daily scale. In future studies, additional thin-sections of *Unio* sp. shells with a known date of death have to be prepared to visualize microincrements and measure the microincrement widths in several years of known date (Subsections 3.4.1 and 4.4.2, Figs. 20 and 21). Knowing the date of death and the water depth in which the bivalves have lived in, environmental datasets with a daily resolution can be correlated with the microincrement data. This would be essential to find the main driving factors for *Unio* sp. growth on a daily scale. Finally, a frequency analysis on the measured microincrement widths could help to identify information on reoccurring signals within the daily growth record.

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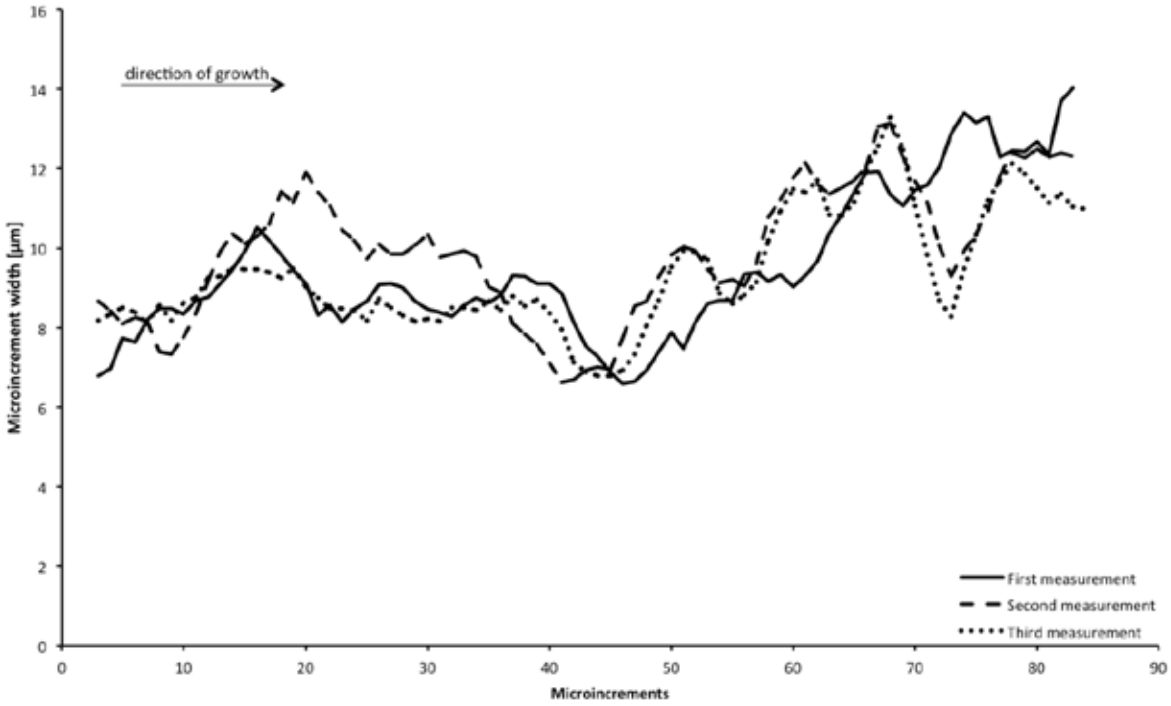
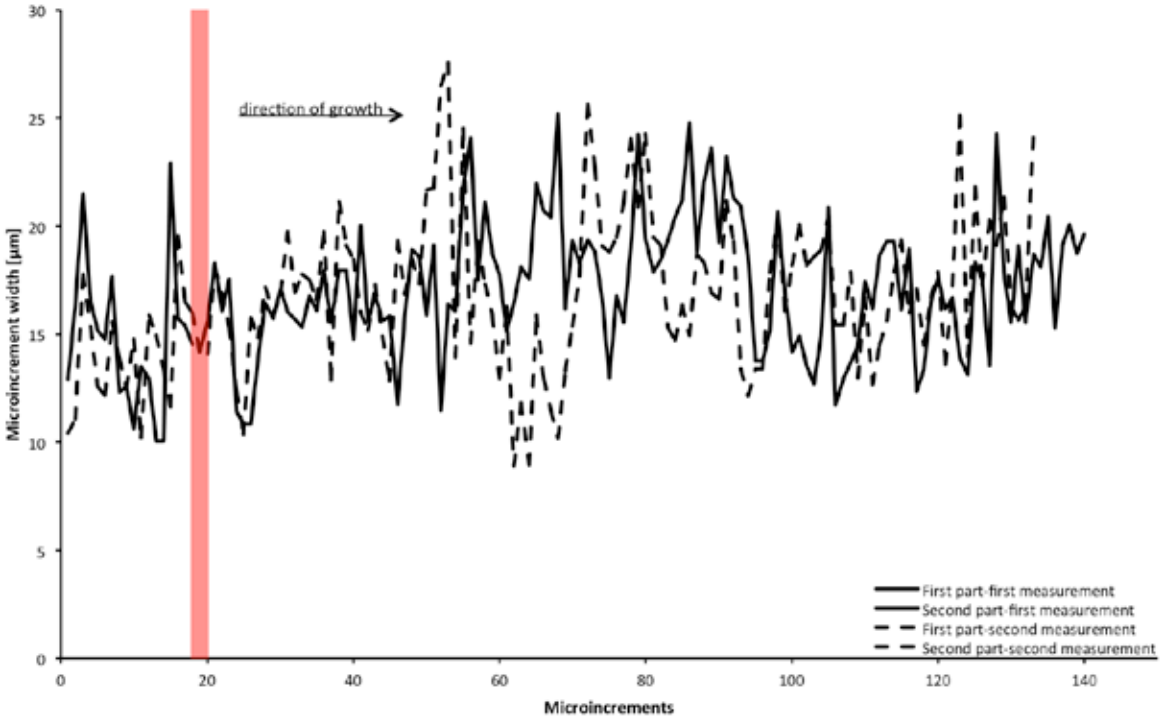
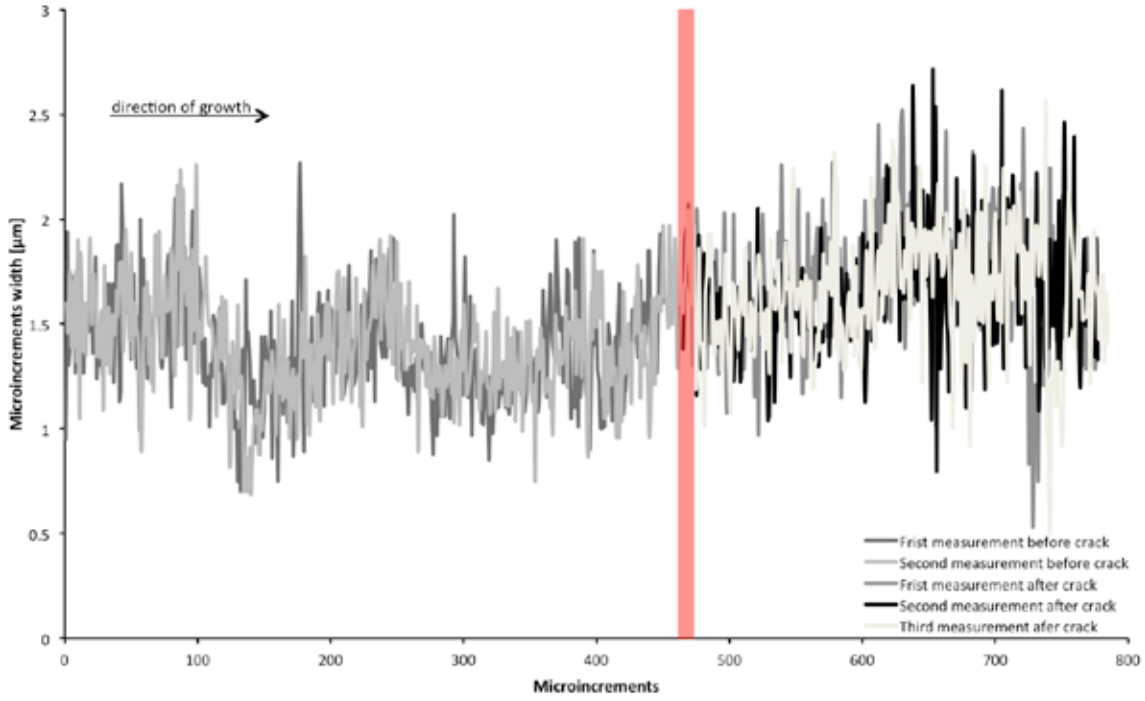
Appendix



Appendix 1: Three measurements of one cutout of the shell of A. islandica (sample-ID: Arc Is 317). This figure is based on the Gaussian filter. The figure with the raw-data is mapped in Subsection 3.4.1 (Fig. 17).



Appendix 2: Showing the growth trend of *A. islandica*. This figure is based in the raw data. The red bar illustrates a break during the measurements (Fig., 18, Subsection 3.4.1).



Appendix 3: Illustration of the microincrement width in one freshwater shell (sample-ID: LB-LaMa-02R). The red bar illustrates a crack which interrupts consistent measurement. The microincrements were measured until the ventral margin. This figure shows the raw data. The Gaussian filter (Fig. 20; Subsection 3.4.2) is based on this figure.