

**Influence of elevated seawater temperature on amphisteginid  
Foraminifera in Maltese waters**

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**A dissertation submitted to the Faculty of Science, University of Malta,  
in part fulfilment of the degree of Master of Science**

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**August 2019**



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*“It is a curious situation that the sea, from which life first arose, should now be threatened by the activities of one form of that life. But the sea, though changed in a sinister way, will continue to exist; the threat is rather to life itself.”*

Rachel Carson, 1960, *The Sea Around Us*

## **Declaration of authenticity**

I, the undersigned, declare that this dissertation is my own original work, except as acknowledged in the text, and that it was carried out under the supervision of Dr Julian Evans and Prof. Patrick J. Schembri. I have clearly indicated where I have used the published or unpublished work of others and I have provided the source of such work. I have acknowledged all main sources of help, and where the work was done jointly with others, I have specified what I have contributed and what has been contributed by others. Any conclusions, suggestions or assumptions are mine unless otherwise stated or attributed.

## **Declaration on ethics**

I declare that I have abided by the University's Research Ethics Review Procedure.

Signature \_\_\_\_\_

Thaís Parreira do Amaral

Date \_\_\_\_\_

## **Declaration on supervision**

The undersigned confirm that this dissertation has been undertaken under their supervision and that they approve of its final submission.

Signature \_\_\_\_\_

Dr Julian Evans

Date \_\_\_\_\_

Signature \_\_\_\_\_

Prof. Patrick J. Schembri

Date \_\_\_\_\_

## Abstract

Sea warming due to climate change might favour the spread of invasive species by expanding their distribution into new areas. The Mediterranean Sea warming and its particular biogeographic history makes it a hotspot for marine invasions, and a case of a successful thermophilic invader in this sea is the marine benthic foraminifer *Amphistegina lobifera*, considered to be of Red Sea origin. The range expansion of *A. lobifera* is likely to trigger changes in ecosystem functions, including the displacement of local foraminiferal species, homogenization of foraminiferal assemblages and drastic alterations in coastal habitats. In the Maltese archipelago, the presence of this alien invasive species could bring about changes to the granulometric characteristics of marine sediments present in bottom habitats, changes which in turn may impact certain benthic species and communities. Elevated temperature has also been identified as causing morphological variations in the shells of benthic Foraminifera, including *A. lobifera*.

The aim of the present study was to use the thermal effluent from the Delimara Power Station, Malta, as a proxy for sea warming in order to assess climate change impacts on the distribution and morphology of the invasive foraminiferan *A. lobifera*. This was made by comparing populations of *A. lobifera* at the impacted site (an inlet receiving thermal effluent) with two geographically similar inlets without thermal effluent (reference sites).

No significant differences in the thickness/diameter ratio, lateral asymmetry and coiling direction of *A. lobifera* were noted between the impacted and reference sites. Overall, most of the specimens in the present study were sinistrally coiled, and tests were significantly larger and with a higher incidence of irregular keels at the impacted than at the two reference sites. Both absolute and relative abundances of *A. lobifera* at the impacted site were significantly different from values of the same attribute recorded from the reference sites, and significant differences were also found in the impact of amphisteginid invasions on the mean sediment grain size. However, no significant differences in sediment sorting values were noted between the impacted and reference sites. These findings match the described westward invasion of *A. lobifera* in the Mediterranean, and indicate that the colonisation of the alien in the studied sites is temperature-driven and possibly displacing local foraminiferal species.

Amphisteginid invasions in Malta will probably intensify as the sea warms up and cause changes in the invaded micro-ecosystem, which is likely to also pose long-term impacts in local meso-ecosystems with significant ecological consequences for presently-occurring marine benthic communities.

## Table of contents

<b>Dedication</b> .....	<b>i</b>
<b>Declaration of authenticity and declaration on ethics</b> .....	<b>ii</b>
<b>Declaration on supervision</b> .....	<b>iii</b>
<b>Abstract</b> .....	<b>iv</b>
<b>Table of contents</b> .....	<b>v</b>
<b>List of appendices</b> .....	<b>vii</b>
<b>List of tables</b> .....	<b>xiii</b>
<b>List of figures</b> .....	<b>x</b>
<b>Acknowledgments</b> .....	<b>xv</b>
<b>Chapter 1: Introduction</b> .....	<b>1</b>
1.1 The foraminiferan <i>Amphistegina lobifera</i> .....	<b>1</b>
1.2 Influences of climate change on <i>Amphistegina lobifera</i> and implications for the Mediterranean Sea.....	<b>4</b>
1.3 Ecological impacts of the <i>Amphistegina lobifera</i> invasion of the Mediterranean.....	<b>9</b>
1.4 The Delimara Power Station and the use of thermal effluents as proxies for climate change impacts.....	<b>12</b>
1.5 Aim and objectives of the present study.....	<b>15</b>
<b>Chapter 2: Material and Methods</b> .....	<b>16</b>
2.1 Study sites.....	<b>16</b>
2.2 Sampling stations.....	<b>17</b>
2.3 Sampling and staining process.....	<b>20</b>
2.4 Subsplitting and laboratory analyses.....	<b>21</b>
2.5 Granulometric analysis.....	<b>23</b>
2.6 Assessment of the proportion of particles in the samples.....	<b>24</b>
2.7 Photographs of <i>Amphistegina lobifera</i> for morphological characteristics.....	<b>25</b>
2.8 Shell morphometry.....	<b>28</b>
2.9 Data analysis.....	<b>31</b>
<b>Chapter 3: Results</b> .....	<b>32</b>
3.1 Comparing absolute and relative abundances of <i>Amphistegina lobifera</i> between the three sites.....	<b>32</b>
3.2 Greatest spiral diameter (GSD).....	<b>35</b>

3.3 Incidence of irregular keel.....	36
3.4 Thickness/diameter (T/D) ratio.....	37
3.5 Coiling direction.....	38
3.6 Lateral asymmetry.....	39
3.7 Aberrant morphologies.....	41
3.8 Mean particle size.....	45
3.9 Particle sorting.....	48
<b>Chapter 4: Discussion.....</b>	<b>54</b>
4.1 Absolute and relative abundances of <i>Amphistegina lobifera</i> and relation to sediment granulometric characteristics.....	54
4.2 The influence of temperature on the spread of <i>Amphistegina lobifera</i> in the analyzed sites.....	57
4.3 The ecological implications of the spread of <i>Amphistegina lobifera</i> in the analyzed sites.....	59
4.4 Greatest spiral diameter (GSD).....	60
4.5 Morphological deformities in <i>Amphistegina lobifera</i> (irregular keel, lateral asymmetry, T/D ratios and severe abnormalities).....	61
4.6 Coiling direction.....	66
4.7 General conclusions.....	67
4.8 Future work.....	68
<b>References.....</b>	<b>69</b>



## List of Appendices

**Appendix A:** Raw data of sampled physico-chemical parameters.

**Appendix B:** Entire dataset for absolute and relative abundance, greatest spiral diameter, thickness/diameter ratio, coiling direction, lateral asymmetry, particle size and sorting.

**Appendix C:** Complete results for all the statistical analyses performed.

**Appendix D:** Entire granulometric analyses results.

## List of tables

**Table 2.2.1** – Geographical coordinates for the sampling stations, the collection depth, and mean temperature (calculated from three readings taken at the time of sampling). Coordinates derived from Google Earth Pro 7.3.2.5776, 2019.

**Table 2.7.1** – Number of photographed specimens per station for assessing greatest spiral diameter (GSD), thickness/diameter ratio (flattening), coiling direction and lateral asymmetry.

**Table 3.1.1** – Total absolute abundance of Foraminifera (individuals/g) for each site. [I = impacted site; R1 = reference site 1; R2 = reference site 2]

**Table 3.1.2** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in absolute abundance of *Amphistegina lobifera* between the impacted and reference sites.

**Table 3.1.3** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in relative abundance of *Amphistegina lobifera* between the impacted and reference sites.

**Table 3.2.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in greatest spiral diameter of *Amphistegina lobifera* between the impacted and reference sites.

**Table 3.3.1** – Counts and percentage abundance of regular and irregular keel morphologies in *Amphistegina lobifera* at the three sites.

**Table 3.3.2** – Chi-square values of the counts of incidence of irregular keel in *Amphistegina lobifera* in the three sites.

**Table 3.4.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in thickness/diameter ratio of *Amphistegina lobifera* between the impacted and reference sites.

**Table 3.5.1** – Chi-square values of the counts of incidence of dextral/sinistral specimens of *Amphistegina lobifera* in the three sites.

**Table 3.6.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in lateral asymmetry ratios in *Amphistegina lobifera* between the impacted and reference sites.

**Table 3.7.1** – The number of severely aberrant *Amphistegina lobifera* at the investigated sites.

**Table 3.7.2** – Description of the different types of severely aberrant morphologies encountered during the present study, and the sites where they occurred. Specimen numbers refer to the examples illustrated in Figure 3.7.2.

**Table 3.8.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in the extent of change (%) in mean sediment grain size due to the presence of *Amphistegina lobifera* between the impacted and reference sites.

**Table 3.9.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in extent of change (%) in sorting ( $\Phi$ ) due to the presence of *Amphistegina lobifera* between the impacted and reference sites.

## List of figures

**Figure 1.1.1** – Live individual of *Amphistegina lobifera*. Retrieved and adapted from Schmidt *et al.*, 2016b. Scale bar: 1mm.

**Figure 1.1.2** – *Amphistegina lobifera* individuals showing different types of morphology. The specimen on the left shows normal morphological characteristics, while the other (center and right photos which are different views of the same individual) shows an irregular keel and lateral asymmetry. Retrieved and adapted from Yanko *et al.*, 1998. Scale bars: 200µm.

**Figure 1.2.1** – Biogeographic distribution of *Amphistegina* spp. in the Mediterranean and northern Red Sea. Retrieved from Langer *et al.*, 2012.

**Figure 1.2.2** – Current biogeographic distribution and abundance of *Amphistegina lobifera* in the Sicily channel (advanced, medium or early stages of spread). Blue arrows indicate the local surface current circulation (Modified Atlantic Water – MAW). Retrieved from Guastella *et al.*, 2019.

**Figure 1.2.3** – Species distribution model for *Amphistegina lobifera* in the Mediterranean Sea under projected climate conditions for 2090–2100. Habitat suitability is marked by the coloured areas (orange as ‘highly suitable’, green as ‘suitable’ and no colour as ‘unsuitable’), and the white triangles represent “*occurrence records for the computation of the species models*”. Retrieved and adapted from Guastella *et al.*, 2019.

**Figure 1.3.1** – Sea bottom abundance of *Amphistegina lobifera* individuals in Turkey, in which some locations show 30–60 cm layers of accumulated tests. Retrieved from Yokes *et al.*, 2014.

**Figure 1.4.1** – Seabed seawater temperature at the impacted site during winter (left) and summer (right) conditions, showing the water flow pattern generated by the thermal discharge (black cross) that guided the distribution of sampling stations in the present study. Warmer colors represent higher temperatures, and the thermal effluent position is marked by the black cross. Retrieved from AIS Environmental Ltd. (2011).

**Figure 2.1.1** – Map showing the location, on the coast of the island of Malta, of the three sites analyzed in this study. Base map from Google Earth Pro 7.3.2.5776, 2019.

**Figure 2.1.2** – Map showing the geomorphological similarity between sites I (Il-Ħofra ż-Żgħira) and R1 (Il-Ħofra I-Kbira), as well as the narrow elongated shape of site R2 (Il-Kalanka Bay). The position of Delimara Power Station between the three bays is marked by the red pin; the thermal effluent from the power station is released into site I. Base map: <https://www.google.com/earth/>, 2019.

**Figure 2.2.1** – Map showing the distribution of the sampling stations at sites I (stations IA – IE) and R1 (stations R1A – R1E). The source of the thermal effluent outfall position is indicated by the red arrow. Base map from <https://www.google.com/earth/>, 2019.

**Figure 2.2.2** – Map showing the distribution of the sampling stations at site R2. Base map from <https://www.google.com/earth/>, 2019.

**Figure 2.4.1** – Photograph showing the splitter device used for unbiased subsampling of the sediment.

**Figure 2.7.1** – Photograph showing the random subsampling device used for splitting stained *Amphistegina lobifera* shells for photographing.

**Figure 2.7.2** – Diagram of the ventral (left) and dorsal (right) view of a sinistral *Amphistegina lobifera* shell. Modified from Hallock & Larsen, 1979.

**Figure 2.7.3** – Ventral views of a (1) dextral specimen and (2) sinistral specimen of *Amphistegina lobifera* collected in the present study. Scale bars: 0.5mm.

**Figure 2.7.4** – Diagram showing the lateral view as defined in the present study (area opposite to the aperture). The aperture is facing away from the observer and is here represented by the dotted circle; the ventral (umbilical) side of the shell is the one on the right (V), and the dorsal (spiral) side on the left (D).

**Figure 2.8.1** – Diagram showing the greatest spiral diameter (red line; GSD) and the greatest spiral diameter perpendicular to the greatest spiral diameter (blue line; SD90) measurements as taken in the present study.

**Figure 2.8.2** – Diagram showing how the measurements to calculate the radius of curvature of the arc representing the sinuous keels of stained *Amphistegina lobifera* were taken. The red line represents the width (W) and the blue line the height (H).

**Figure 2.8.3** – Diagram showing the main range of variation present in the different categories of regular and irregular keel considered in this study (lateral view of shells).

**Figure 3.1.1** – Mean absolute abundance of *Amphistegina lobifera* (ind./g) per station in the three studied sites. Error bars represent +SD. [I = impacted site; R1 = reference site 1; R2 = reference site 2].

**Figure 3.1.2** – Mean relative abundance of *Amphistegina lobifera* (%) per station in the three studied sites. Error bars represent +SD.

**Figure 3.2.1** – Mean values of greatest spiral diameter (mm) of *Amphistegina lobifera* per station in the three studied sites. Error bars represent +SD.

**Figure 3.4.1** – Mean values of thickness/diameter ratio of *Amphistegina lobifera* per station in the three studied sites. Error bars represent +SD.

**Figure 3.5.1** – Proportion (%) of coiling direction (dextral/sinistral) in *Amphistegina lobifera* per station.

**Figure 3.6.1** – Mean values of lateral asymmetry ratios of *Amphistegina lobifera* per station in the three studied sites. Error bars represent +SD.

**Figure 3.6.2** – Photomicrographs of the lateral views of *Amphistegina lobifera* tests showing the two outlier specimens presenting a 'D shape' (1a and 1b), a specimen that is slightly asymmetrical (2) and a symmetrical one (3). Scale bar: 0.5mm.

**Figure 3.7.1** – Photomicrograph of the dorsal view of a stained, non-deformed specimen of *Amphistegina lobifera* with typical morphology. Scale bar: 0.5mm.

**Figure 3.7.2** – Photomicrographs of Rose Bengal stained *Amphistegina lobifera* specimens found in the studied sites which show severe morphological deformities. The deformities are described in Table 3.7.2. Scale bars: 0.5mm.

**Figure 3.8.1** – Mean particle size ( $\mu\text{m}$ ) of the different categories analyzed per site. Error bars represent +SD.

**Figure 3.8.2** – Mean particle size ( $\mu\text{m}$ ) of the different categories analyzed per station in the impacted site. Error bars represent +SD.

**Figure 3.8.3** – Mean particle size ( $\mu\text{m}$ ) of the different categories analyzed per station in the reference site 1. Error bars represent +SD.

**Figure 3.8.4** – Mean particle size ( $\mu\text{m}$ ) of the different categories analyzed per station in the reference site 2. Error bars represent +SD.

**Figure 3.8.5** – Mean extent of change in particle size (%) due to the presence of *Amphistegina lobifera* per station in the three analyzed sites. Error bars represent +SD.

**Figure 3.9.1** – Mean sorting coefficient ( $\Phi$ ) of the different analyzed categories per site. Error bars represent +SD.

**Figure 3.9.2** – Mean sorting coefficient ( $\Phi$ ) of the different analyzed categories in the impacted stations. Error bars represent +SD.

**Figure 3.9.3** – Mean sorting coefficient ( $\Phi$ ) of the different analyzed categories per station in the reference site 1. Error bars represent +SD.

**Figure 3.9.4** – Mean sorting coefficient ( $\Phi$ ) of the different analyzed categories per station in the reference site 2. Error bars represent +SD.

**Figure 3.9.5** – Mean extent of change in sorting (%) due to the presence of *Amphistegina lobifera* per station in the three three analyzed sites. Error bars represent +SD.

**Figure 3.9.6** – Extent of change in sediment sorting (%) in the three replicates of station IC, illustrating the natural variability found between the samples' replicates.



## Acknowledgements

I would firstly like to express my deepest acknowledgement to the Government of Malta, Ministry for the Environment, Sustainable Development and Climate Change, to whom I am indebted for the award of a scholarship to read for a master's degree in collaboration with the University of Malta through the Climate Action Scholarships. Special thanks also to all the University of Malta's staff who have been involved in making this project and the opportunity of coming to Malta possible since its early beginning.

Secondly, my profound gratitude to my supervisors, Dr Julian Evans and Prof. Patrick J. Schembri, who have kindly and carefully guided me throughout this year and with whom I have gained much knowledge and experience for my career. Many thanks also to all the colleagues and staff from the Department of Biology at the University of Malta, who helped me in many different ways – from field collection to general daily lab activities and issues.

This project has also been assisted by Ms. Micaela Cassar, who kindly shared data from her surveys at Il-Ħofra ż-Żgħira, and by Dr. Hallock Muller and Dr. Yanko who also provided help by sharing some of their research work.

I'm particularly grateful for the valuable help given by Lisa, whose assistance was greatly appreciated and essential to the outcome of this project. Special thanks also to my international friends spread throughout and coming from different parts of the planet. Merci, danke, oshey, seryer sa, nandri, grazzi... there are too many different languages, so simply: thank you all.

Finally, my sincere thanks to all my family members and friends from Brazil who have, mostly virtually but yet thoroughly, supported another adventure of mine in so many different and beautiful ways. Thanks for listening, advising, caring and coping. Muito obrigada mesmo.

Thaís

# 1. Introduction

## 1.1 The foraminiferan *Amphistegina lobifera*

Foraminifera is a group of organisms currently classified as a phylum in the kingdom Chromista (WoRMS, 2019). Such unicellular eukaryotes possess a cytoplasm supported by a test/shell (skeletal component of a foraminifer) that can be made out of several materials, and which has a hole ('foramen') that connects its chambers (BouDagher-Fadel, 2008; Hottinger, 2006). They can be aquatic, marine, planktonic or benthic, and the latter includes a group informally referred to as 'larger benthic Foraminifera' (BouDagher-Fadel, 2008). This group is comprised of 14 Orders which include species that are not always necessarily larger than other benthic Foraminifera, but which actually possess complicated internal structures (a feature that defines the group), and that might offer housing for algal endosymbionts (BouDagher-Fadel, 2008). Still, some larger benthic Foraminifera can exceed a volume of 3mm<sup>3</sup> (Ross, 1974), a large size compared to other foraminiferan groups, and most are mixotrophic, being able to feed on particulate food and at the same time host endosymbiotic algae that photosynthesize (Hallock, 1981).

In symbiont-bearing larger benthic Foraminifera, most of the energy captured through organic matter ingestion is used for the host's reproduction and growth (Hallock, 2000), while energy from photosynthesis is directed to the host's respiration (Falkowski *et al.*, 1984, as cited in Hallock, 2000). Hosting symbionts in their complex internal test structures enables symbiont-bearing larger benthic Foraminifera to increase their calcification rates since the energy acquisition through photosynthesis is higher than through heterotrophy; this condition is favourable especially in oligotrophic environments like tropical coral reefs (Hallock, 1981). The symbionts also produce amino acids that photo-protect the host against ultraviolet (UV) irradiation (Jokiel & York, 1981, as cited in Hohenegger, 2009).

The species *Amphistegina lobifera* Larsen, 1976 (Hayward *et al.*, 2019a) is a symbiont-bearing larger benthic foraminiferan that hosts diatoms as endosymbionts (Lee, Reimer & McEnergy, 1980), and occurs mostly in subtropical and tropical coastal and shallow areas (Langer & Hottinger, 2000). The current distribution of the genus *Amphistegina* is limited by the 14°C winter isotherm (Zmiri *et al.*, 1974; Larsen, 1976; Langer & Hottinger, 2000).

Compared to other amphisteginids, *A. lobifera* shows a very round test (the skeletal component of a foraminifer; Figure 1.1.1) for protecting the symbionts from light and water motion (Hallock, 1979). The typical reproduction in Foraminifera usually involves an alternation between asexual reproduction via multiple fission, in which the parent's test is discarded after the protoplasm is divided into smaller portions for generating juveniles; and reproducing sexually through the production of gametes (Loeblich and Tappan, 1964, as cited in Hallock, 1985). Sexual reproduction involves a planktonic life stage due to the production of flagellated gametes (Hallock, 1981). Foraminifera in general, therefore, usually reproduce only once, and the parents die after the young are generated. Typical generation times for *A. lobifera* range from 6–12 months (Hallock, 1999), and it usually takes around 30 days for an amphisteginid embryo to reach 0.5 mm in diameter (Hallock, 1985). The life span of amphisteginids varies between four and twelve months (Hallock, 1981; Hallock *et al.*, 1995; Triantaphyllou *et al.*, 2012). Reproduction in *Amphistegina* spp. occurs in individuals >1 mm. In each reproductive event, *A. lobifera* generates up to 2,000 young while *Amphistegina lessonii*, another commonly studied amphisteginid species, produces several hundred young (Hallock, 1985). A population of *A. lobifera* from the South of Greece is known to probably reproduce mainly during summer, in which both sexual and asexual reproduction occur simultaneously (Triantaphyllou *et al.*, 2012).

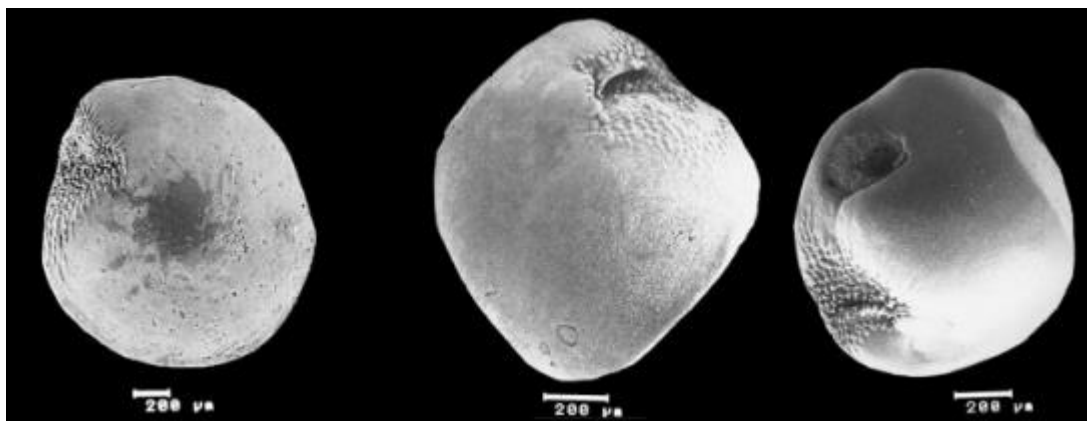


**Figure 1.1.1** – Live individual of *Amphistegina lobifera*. Retrieved and adapted from Schmidt *et al.*, 2016b. Scale bar: 1mm.

The depth distribution of *Amphistegina* spp. ranges from 0–130 m (Hallock, 1985). While *A. lobifera* lives in turbulent and shallow waters, mostly at <20 m (Hallock, 1985; Triantaphyllou *et al.*, 2012 and references therein), *A. lessonii* has a slightly deeper distribution, between 5–30 m (Hallock, 1985). *A. lobifera* is found predominantly on coarse sediment and phytal substrata (Hogenerger, 1994; Triantaphyllou *et al.*, 2012).

The typical morphology of *A. lobifera* comprises an unequally biconvex test, with the spiral side being more flattened than the umbilical side, which is more convex; the margin of the test shows a carina (defined as a “*peripheral thickening of the shell*” in Hottinger, 2006, p. 38) and the last whorl, close to the aperture, is covered by papillae (Caruso & Cosentino, 2014a). The carina is also often referred to as a ‘keel’, as well as the spiral side as being the ‘dorsal’ one, and the umbilical as ‘ventral’. Young specimens do not show the evident lobate septa that characterize the species, making them very similar to *A. lessonii* (Hogenerger *et al.*, 1999). *Amphistegina lobifera* individuals can be sinistrally or dextrally coiled (Hallock & Larsen, 1979).

General morphological variations (e.g. size and test ornamentation) in benthic Foraminifera can be related to different environmental factors which include temperature, salinity, light, water motion, nutrition, pollution, substratum, etc. (Boltovskoy, Scott & Mediolli, 1991). Such variations, sometimes also referred to as ‘deformities’, are described in the literature as related especially to heavy metal pollution, since benthic Foraminifera are commonly used for pollution monitoring (Alve, 1991; Sharifi, Croudace & Austin, 1991; Yanko, Kronfeld & Flexer, 1994). Intraspecific variations in the morphology of benthic Foraminifera also seem to be related to elevated temperature (Boltovskoy *et al.*, 1991). In *A. lobifera*, some described deformities include lateral asymmetry and an irregular keel (Yanko, Ahmad & Kaminski, 1998; Figure 1.1.2), besides other kinds of morphological variations like aperture abnormalities, segment loss in the peripheral margin, dorso-ventral asymmetry, etc. (Sen Gupta, 2007). An increase in sediment concentration of cadmium, for instance, has been described to significantly influence *A. lobifera* morphological characteristics, leading to a higher proportion of deformities, specifically to an ‘irregular keel development’ and ‘lateral asymmetry’ (Yanko *et al.*, 1998).



**Figure 1.1.2** – *Amphistegina lobifera* individuals showing different types of morphology. The specimen on the left shows normal morphological characteristics, while the other (center and right photos which are different views of the same individual) shows an irregular keel and lateral asymmetry. Retrieved and adapted from Yanko *et al.*, 1998. Scale bars: 200µm.

Flattening has also been described as a morphological deformity in *A. lobifera* (see review by Boltovskoy *et al.*, 1991). One possible way of measuring flattening is through the ‘thickness to diameter ratio’ (Hallock, Forward & Hansen, 1986), in which higher ratios represent thicker (less flat) shells. For this, some workers consider thickness as referring to the lamellar thickness<sup>1</sup> (e.g. Hallock & Hansen, 1979; Hallock, 1981), but others use the concept of thickness as being the minimum diameter of the shell (i.e., the lateral one; Mateu-Vicens, Hallock & Brandano, 2009). Either way, the ratios are related since the latter is directly influenced by the secondary lamellae thickness (Hallock & Hansen, 1979; Hallock *et al.*, 1986). In general, *A. lobifera* tests are very robust, with typical thickness/diameter ratios reaching >0.6 (Hallock, 1979).

## **1.2 Influences of climate change on *Amphistegina lobifera* and implications for the Mediterranean Sea**

Anthropogenic climate change is altering ocean ecosystems and causing ecological impacts on food web dynamics, species distributions, incidence of diseases, ocean productivity and abundance of habitat-forming species (Hoegh-Guldberg & Bruno, 2010). One of the overall biological consequences of climate change is that it might

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<sup>1</sup> ‘Lamellar thickness’ refers to the secondary lamellae thickness, which cover the primary lamellae that constitute the lateral chamber walls (BouDagher-Fadel, 2008).

favour some invasive<sup>2</sup> species (Dukes & Mooney, 1999). For example, seawater warming in the Mediterranean (Lejeusne *et al.*, 2010) is facilitating the spread of thermophilic alien<sup>3</sup> species (Raitsos *et al.*, 2010) and contributing to increasing the Mediterranean's susceptibility to alien introductions (Bianchi, 2007). In fact, the Mediterranean Sea is considered as a hotspot for climate change-driven marine invasions (Galil, Marchini & Occhipinti-Ambrogi, 2018).

Being one of the areas in the world more severely impacted by the presence of alien species (Boudouresque, Ruitton & Verlaque, 2005), the Mediterranean hosts around 736 multicellular non-invasive species recorded from the 1800s up to date, which would then contribute to ca 4.3% out of its more than 17,000 described marine species (Galil *et al.*, 2018). Under this scenario, some authors propose that the Mediterranean is going through a 'meridionalisation' process, in which the south of the sea is becoming populated by subtropical species (e.g subtropical Atlantic species mixed with Red Sea immigrants), and the north by the thermophilic species that currently inhabit the east and south of the Mediterranean (Lejeusne *et al.*, 2010). At the same time, this same process of the increase in the distribution and abundance of tropical and subtropical species in the Mediterranean Sea has also been referred to as 'tropicalization' (Bianchi & Morri, 2003). As a matter of fact, these terms, as well as others that have been suggested in the literature (i.e subtropicalization and septentrionalization), are all used to describe the same phenomenon that is currently taking place in the Mediterranean Sea (Bianchi *et al.*, 2018): the climate warming-driven establishment of thermophilic species into new areas out of their original range (Canning-Clode & Carlton, 2017). The future continuation of the present ocean warming would then likely lead to a homogenization of the currently distinct biotas in the Mediterranean sub-basins (Bianchi *et al.*, 2012).

The Suez Canal, a man-made waterway constructed in Egypt in 1869 that connects the Red Sea to the Mediterranean, is the main vector for the introduction of non-

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<sup>2</sup> An invasive species can be described as “*an established species whose population has undergone an exponential growth phase and may threaten the diversity or abundance of native species and the ecological stability of the impacted ecosystem and which may also threaten economic activities dependent on these ecosystems, and/or human health*” (Evans, Barbara & Schembri, 2015, p. 228).

<sup>3</sup> The definition of alien species can be summarized as follows: “*species or infraspecific taxa, inclusive of parts, gametes or propagules, that may survive and subsequently reproduce and spread outside of their historically known range (geographical area occupied naturally) and beyond their natural dispersal potential (due to minor climatic oscillations) as a result of deliberate or accidental introduction by humans*” (Evans *et al.*, 2015, p. 227).

indigenous species into the Mediterranean Sea, being followed by shipping and mariculture (Galil *et al.*, 2018). The opening of the Suez Canal allowed different taxa to migrate between the Red and the Mediterranean Sea (mostly species coming from the Red Sea and then invading the Mediterranean), in a phenomenon termed 'Lessepsian Migration' (Por, 1978).

Amphisteginids are an example of a problematic invasive group in the Mediterranean Sea, since climate change is known to promote their spread into new areas outside their original range. Two species of *Amphistegina* are known to occur in the Mediterranean Sea at present: *A. lobifera* Larsen, 1976 and *A. lessonii* d'Orbigny in Guérin-Méneville, 1832 (Langer *et al.*, 2012; Hayward *et al.*, 2019b), the former being from Indo-Pacific and Atlantic origin (Langer and Hottinger, 2000), and the latter is considered as cryptogenic (Guastella *et al.*, 2019).

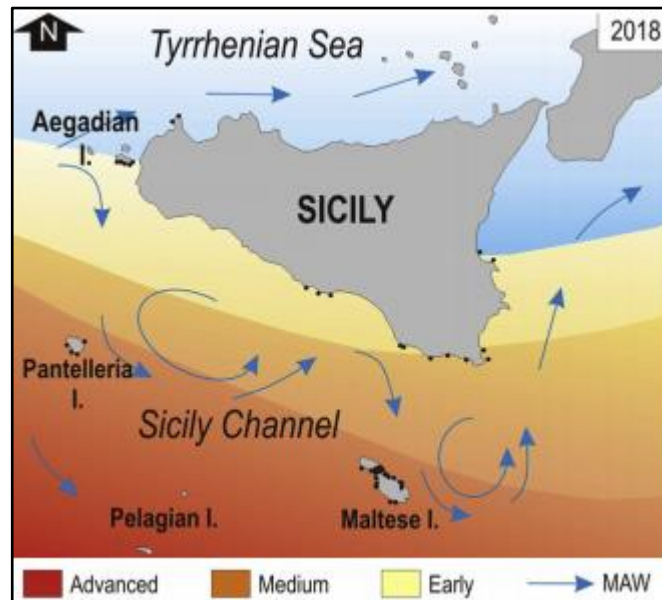
Both *A. lobifera* and *A. lessonii* are generally accepted to have entered the Mediterranean Sea through the Suez Canal and to be successful Lessepsian immigrants (Weinmann *et al.*, 2013a; Caruso & Cosentino, 2014b; Guastella *et al.*, 2019). However, findings of *Amphistegina* spp. in the Mediterranean from the Miocene and Pliocene (Di Bella, Carboni & Pignatti, 2005) made some authors suggest that amphisteginids were originally native to the Mediterranean, having been temporarily displaced during the Messinian Salinity Crisis and being now successful returnees to the Mediterranean Sea (Langer *et al.*, 2012). It has also been suggested that, instead of the Suez Canal, some alien benthic foraminifer species (including *A. lobifera* and *A. lessonii*) might have actually entered the east Mediterranean via the Arabian Gulf-Mesopotamia Basin or through a fault line between the Gulf of Aqaba-Dead Sea (Meric *et al.*, 2018).

Regardless of the debate if *Amphistegina* spp. are Lessepsian re-colonizers of the Mediterranean or true aliens, as well as their pathway of arrival into the Mediterranean, currently the genus occupies a wide area in the eastern Mediterranean, having already reached central areas too (Langer *et al.*, 2012; Guastella *et al.*, 2019). Figure 1.2.1 shows Mediterranean records of *Amphistegina* spp. up to 2012, and Figure 1.2.2 the new records of *A. lobifera* in the Strait of Sicily from 2018. Differently from *A. lessonii*, whose invasion of the eastern Mediterranean has not yet been significant (Titelboim *et al.*, 2019), *A. lobifera* seems to be spreading westwards in the Mediterranean (Yokes, Meric & Avsar, 2007; Koukousioura, Dimiza

& Triantaphyllou, 2010; Langer *et al.*, 2012; Yokes *et al.*, 2014; Guastella *et al.*, 2019). At the same time, the spread direction of the species in the Sicily Channel is probably related to the local surface current circulation of the Modified Atlantic Water (Guastella *et al.*, 2019; Figure 1.2.2).



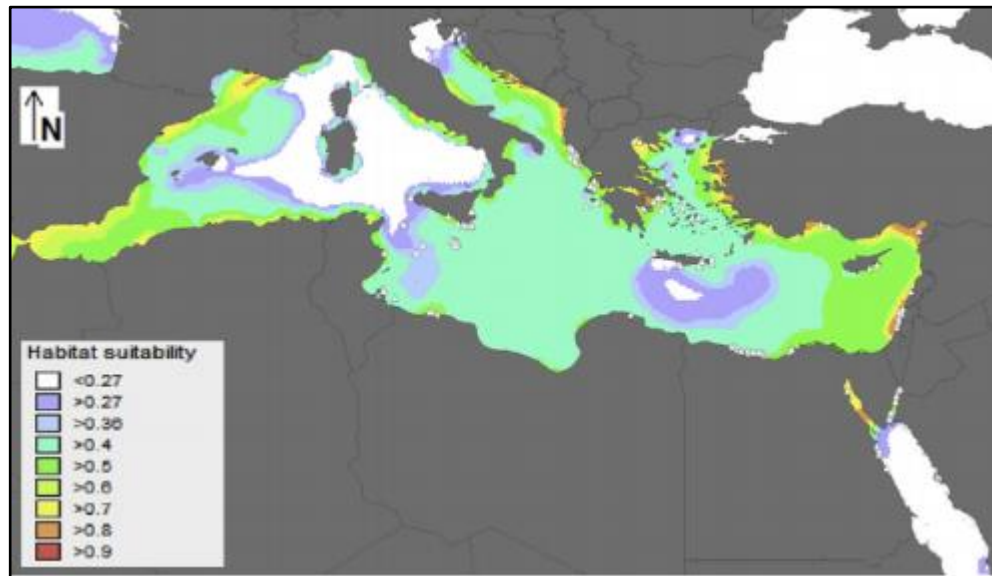
**Figure 1.2.1** – Biogeographic distribution of *Amphistegina* spp. in the Mediterranean and northern Red Sea. Retrieved from Langer *et al.*, 2012.



**Figure 1.2.2** – Current biogeographic distribution and abundance of *Amphistegina lobifera* in the Sicily channel (advanced, medium or early stages of spread). Blue arrows indicate the local surface current circulation (Modified Atlantic Water – MAW). Retrieved from Guastella *et al.*, 2019.



Habitat suitability for *Amphistegina* spp. is expected to widely increase not only in the Mediterranean Sea (Weinmann *et al.*, 2013a; Guastella *et al.*, 2019; Figure 1.2.3), but also in the Atlantic and Indo-Pacific oceans (Weinmann *et al.*, 2013b). With this, the distribution of amphisteginids into new areas, including the western Mediterranean Sea, is likely to become even more widespread.



**Figure 1.2.3** – Species distribution model for *Amphistegina lobifera* in the Mediterranean Sea under projected climate conditions for 2090–2100. Habitat suitability is marked by the coloured areas (orange as ‘highly suitable’, green as ‘suitable’ and no colour as ‘unsuitable’), and the white triangles represent “*occurrence records for the computation of the species models*”. Retrieved and adapted from Guastella *et al.*, 2019.

In the Maltese archipelago, *A. lobifera* was recorded for the first time in 2007 (Yokes *et al.*, 2007). Both *A. lobifera* and *A. lessonii* have been recorded in Maltese waters (Caruso & Cosentino, 2014a), but the method of the arrival of amphisteginids in the islands is yet uncertain. It might have taken place by the carrying of juveniles in ballast water, as well as by their predation and consequent spread by non-indigenous fish (Guy-Haim *et al.*, 2017; Agius, 2018).

However, it is still noteworthy to mention that climate change might also cause negative impacts on amphisteginids. Often referred to as the ‘evil twins’ of climate change (Kawahata *et al.*, 2019), ocean acidification and global warming are known to affect the holobiont health and growth in symbiont-bearing larger benthic Foraminifera (Doo *et al.*, 2014). *Amphistegina lobifera* shows a reduction in growth and in

photosynthesis at 32°C (Schmidt *et al.*, 2016a; Schmidt *et al.*, 2016b). Bleaching under elevated temperature has also been recorded for the species (Prazeres, Uthicke & Pandolfi, 2016).

Finally, another impact of climate change on amphisteginids is that elevated seawater temperature may affect morphological features of the test. With an increase in temperature, test size and growth rate are known to decrease (Prazeres & Pandolfi, 2016; Schmidt *et al.*, 2016a). Changes in coiling direction have also been reported (Muller, 1977). Intraspecific test morphological alterations are also reported in benthic Foraminifera under high temperature conditions, causing shell deformities (Boltovskoy *et al.*, 1991).

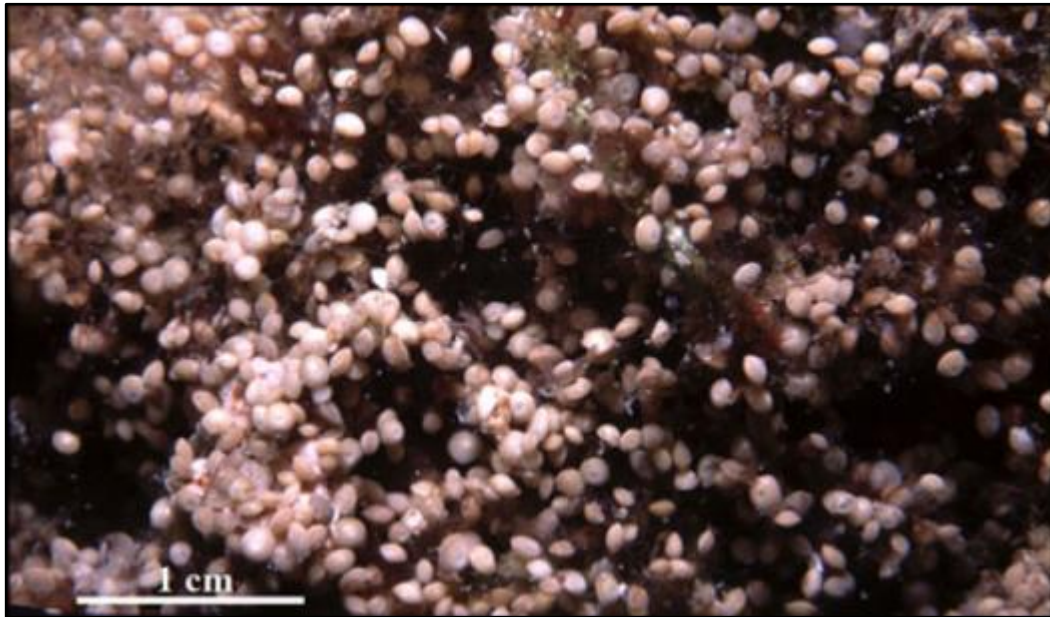
### **1.3 Ecological impacts of the *Amphistegina lobifera* invasion of the Mediterranean**

Larger benthic Foraminifera have a significant contribution to carbonate production in coral reefs (Langer, 2008) and can contribute up to 60% of the contemporary sand mass in some reef islands, being therefore important to reef island sand supplies especially under the current scenarios of changes in carbonate production and sea level rise (Dawson, Smithers & Hua, 2014). Amphisteginid Foraminifera play an important role in carbonate grain production, and their invasion and establishment in new areas leads to higher calcium carbonate accumulation (Mouanga, 2017).

Besides their importance in coral reef ecosystems globally, amphisteginids have been recorded to modify the substratum (Langer *et al.*, 2013) and to alter the sediment composition from mostly siliceous to carbonate (Langer & Mouanga, 2016). In the long term, the areas impacted by their invasion could suffer drastic changes in their sediment structure. This impact is of particular interest in the Mediterranean Sea, where they have been recorded to drastically change the coastal habitats (Meric *et al.*, 2002).

The local dominance of *A. lobifera* influences the stability of shallow habitats and the composition and production of beach sediments (Mouanga & Langer, 2014). Considered as one of the 100 worst invasive species in the Mediterranean (Streftaris & Zenetos, 2006), the species is already changing coastal sediments and habitats in

the Eastern Mediterranean, for example, through the high abundance of their granule-sized tests (Yokes & Meric, 2004; Streftaris & Zenetos, 2006; Figure 1.3.1).



**Figure 1.3.1** – Sea bottom abundance of *Amphistegina lobifera* individuals in Turkey, in which some locations show 30–60 cm layers of accumulated tests. Retrieved from Yokes *et al.*, 2014.

The light conditions, quality and movement of water, sediment accumulation and erosion rates might also be influenced by the establishment of invasive species (Wallentinus & Nyberg, 2007). In Malta, rocky bottoms could accumulate sediment in the form of dead foraminiferal tests, and this could affect the local benthic community structure with regards to attachment and burrowing surfaces. Smothering of the rocky bottom with sediment layers (e.g. a hard bottom changing to a sandy one) could also take place, and this would affect the species that are able to survive in this environment; soft bottom species would probably become more common. At the same time, sediment granulometric characteristics could change, becoming finer or coarser after the invasion of the foraminiferans and this could affect the epiphytic species living on the sediment.

Most Maltese rocks and sediments are carbonates or have a high carbonate content (Pedley, House & Waugh, 1976), meaning that the addition of carbonate amphisteginid shells probably would not significantly influence the local chemical environment. However, as discussed above, amphisteginids are considered as true ecosystem engineers (Langer *et al.*, 2012; Mouanga & Langer, 2014), and their mass

spread can still influence the physical environment through changes in granulometric characteristics and consequently the resources and conditions for other organisms in Maltese waters.

Moreover, non-indigenous species can also modify the habitats they invade by altering ecological interactions, e.g. competition for resources, places to settle and spawn, predation interactions, contraction of available niches, etc. (Wallentinus & Nyberg, 2007). Therefore, the implications of the spread of *A. lobifera* into new areas comprise not only physico-chemical changes in the sediment structure, but also modifications to ecological interactions.

Invasive species can adapt quickly to new environments and act as competitors, prey, consumers or disturbers (Mooney & Cleland, 2001), and *A. lobifera* is one example of a successful invader in the Mediterranean Sea (Triantaphyllou *et al.*, 2012). The food chain impact of benthic Foraminifera in general involves the fact that they are ingested by a wide variety of organisms including polychaetes, holothurians, gastropods, etc. (Culver & Lipps, 2003 and references therein). *Amphistegina* spp. can also be preyed on by other foraminiferans, such as *Floresina amphiphaga* (Hallock *et al.*, 1998). Regarding *A. lobifera* specifically, their invasion of the Mediterranean Sea is also related to their predation by the non-indigenous fish *Siganus luridus* Ruppel, 1829. A live passage through the fish's gut enables *A. lobifera* to spread along the same biogeographic areas as the invasive fish (Guy-Haim *et al.*, 2017). The siganid *S. luridus* has been described in the literature to having been present in Maltese waters at least since 2002 (Schembri, Deidun & Falzon, 2012) and, therefore, might have contributed to the spread of *A. lobifera* around the islands. More importantly, this highlights the complexity of potential pathways, which have contributed to the dissemination of *A. lobifera* in the Mediterranean Sea and in the Maltese waters, as well as the complex ecological interactions affected by the presence of the species.

Amphisteginid invasions also displace local foraminiferal species, leading to a homogenization of foraminiferal faunas (Langer *et al.*, 2012; Mouanga & Langer, 2014). In the eastern Mediterranean, amphisteginid invasions preferentially impact other symbiont-bearing larger benthic Foraminifera (like *Peneroplis pertusus* and *P. planatus*), but also other taxa that are epiphytic too and, therefore, compete for the same microhabitat as *Amphistegina*; the latter includes “*Rosalina*, *Discorbina*, *Asterigerinata*, *Ammonia*, *Lobatula*, *Eponides*, *Cibicides* and *Patellina*” (Mouanga &

Langer, 2014, p. 147). At the same time, smaller non-symbiont-bearing Foraminifera belonging to the order Miliolida are apparently less affected by amphisteginids' invasion, which might be due to the wider range of microhabitats occupied by them (epiphytic, epifaunal, infaunal; Mouanga & Langer, 2014). Hence, *Amphistegina* spp. seem to displace mainly other species that also host endosymbionts and share similar microhabitats, and some of the ecological functions played by them might then become restricted to the amphisteginids. At the same time, some workers suggest that the species' successful adaptation to new environments might be related to the availability of vacant niches for symbiont-bearing perforate foraminifers (Triantaphyllou *et al.*, 2012). Besides that, regardless if the native foraminiferal assemblage has low or high diversity, both situations seem to be equally vulnerable to amphisteginid invasions and, thus, the relation between ecosystem functioning and biodiversity is negatively impacted, with the communities becoming more susceptible to environmental stress after the invasion (Mouanga & Langer, 2014).

The Mediterranean is expected to have an increase in seawater temperature between 2°C and 4°C from 2071–2100 compared to the period 1990–2019 (Hertig & Jacobeit, 2007). Concomitantly, the amphisteginid dispersal's impacts on ecosystem functioning are closely related to the extent of time of their presence, gradually increasing as their range expansion spreads (Mouanga & Langer, 2014). With this, as discussed in section 1.1 and 1.2, in spite of the recorded negative physiological impacts of climate change on symbiont-bearing larger benthic Foraminifera (including, therefore, *A. lobifera*), if there is no change in the current trend of global climate change, the spread of amphisteginids will expand further. This, in turn, will likely lead to an increase in amphisteginid abundance, with more pronounced impacts posing complex ecological consequences on the native ecosystems populated by these invasive foraminiferans.

#### **1.4 The Delimara Power Station and the use of thermal effluents as proxies for climate change impacts**

A thermal effluent can be described as “a discharge of water at elevated temperature, which can cause a drastic change in the native environment” (Sciberras, 2015, p. 3), being responsible for causing severe environmental and ecological changes in its impacted areas (Walkuska & Wilczek, 2010). Due to the presence of elevated temperature in the thermal plume generated by power stations, thermal effluents can

be used as general proxies for assessing possible climate change impacts on biological communities.

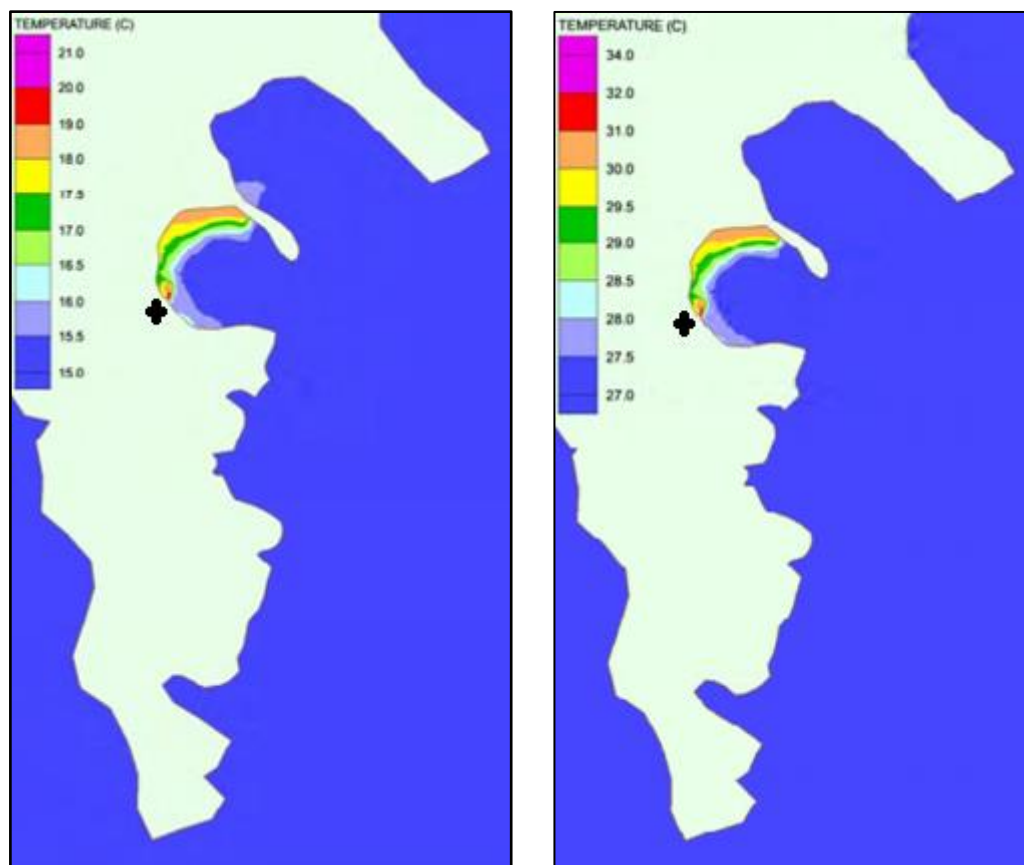
The Delimara Power Station, located at Marsaxlokk Bay (Malta), releases a thermal effluent at Il-Ħofra ż-Żgħira bay, which is known to reach higher seawater temperatures both at the surface and close to the seabed of the bay when compared to background seawater (Figure 1.4.1; AIS Environmental Ltd., 2011). The seabed conditions at the site were predicted to reach between 27–32°C during summer and 15–20°C during winter after an increase in the generating capacity of the power station, meaning a rise of 8°C directly at the outfall (AIS Environmental Ltd., 2011).

At the impacted site, the mean seawater surface temperature is known to vary between 16–18°C in March and from 18–21°C in May, and for both months the temperatures also do not cool below 15°C (Micaela Cassar, *personal communication*, 2018). According to the Delimara Power Station permit conditions, the temperature at the main outfall should not exceed the annual average limit of 8°C above ambient, and it was reported as just 0.7°C higher in 2017 (IPPC Permit for Delimara Power Station, 2017). However, field data from different monitoring studies reveal not only higher values but also fluctuations in the temperature readings of the bay, which may vary depending on the day or season of data collection. For instance, while in the present study the temperatures measured at the impacted stations ranged between 22–23°C (being of 25°C directly at the outfall), data collected two weeks previously (October, 2018) shows that in some areas in Il-Ħofra ż-Żgħira the seawater surface temperature was around 29°C (Micaela Cassar, *personal communication*, 2018). Moreover, previous surface temperatures of ca 26–29°C and bottom temperatures of ca 23–28°C (depths varying from 2.2–6 m; Gatt, 2006) have also been described. Modelling studies showed that, after the increase in discharge at the power station, the seabed temperatures were predicted to reach between 15–20°C during winter and 27–32°C during summer, with the highest temperatures being expected right at the outfall and with colder waters occurring according to the clockwise circulation pattern in the bay (AIS Environmental Ltd., 2011). Finally, more recent recordings in 2014 showed temperature readings that reached up to 28–30°C in August and September (Sciberras, 2015).

Hence, there is probably a daily/small temporal scale fluctuation in the seawater surface temperature in the bay, even though overall it has been described as higher

than adjacent waters (ca 1–2°C, as described above). This becomes evident especially when comparing the impacted site to Il-Ħofra l-Kbira, a bay that is very commonly used as a reference site in research due to its geomorphological similarities with Il-Ħofra ż-Żgħira.

Considering the expected increased temperature in the Mediterranean Sea of 2°–4°C by the end of the century (Hertig & Jacobeit, 2007), the above mentioned temperatures found at the impacted site make it a suitable proxy for assessing some potential impacts on marine ecosystems resulting from climate change-related elevated seawater temperatures. Ecological consequences of the outfall in the bay receiving the discharge have been thoroughly described in the literature and include, for example, alterations in macroalgae and macrofauna community structures (Micallef, 2001; Gatt, 2006; Sciberras, 2015; Agius, 2018).



**Figure 1.4.1** – Seabed seawater temperature at the impacted site during winter (left) and summer (right) conditions, showing the water flow pattern generated by the thermal discharge (black cross) that guided the distribution of sampling stations in the present study. Warmer colors represent higher temperatures, and the thermal effluent position is marked by the black cross. Retrieved from AIS Environmental Ltd. (2011).

## 1.5 Aim and objectives of the present study

Based on the issues described above, the aim of the present study was to use the thermal effluent at Il-Ħofra ż-Żgħira, compared to two reference sites without thermal effluent, to investigate the influence of elevated seawater temperature on the invasive foraminiferan *A. lobifera* in order to better understand how climate change may affect the ecology of the species, its spread and ecosystem effects.

To achieve this, the present study comprised the following objectives:

- To assess possible differences in the absolute abundance (as number of individuals per unit mass of sediment) of *A. lobifera* between the impacted and reference sites;
- To delineate the role played by elevated seawater temperature on the distribution of *A. lobifera* at each study site;
- To estimate the relative abundance of *A. lobifera* at each site, in relation to other native foraminiferal species;
- To determine the possible role of temperature on *A. lobifera* shell morphology;
- To explore the impacts of *A. lobifera* invasion on sediment granulometric characteristics, evaluating if such impacts were enhanced by elevated seawater temperature.



## 2. Material and Methods

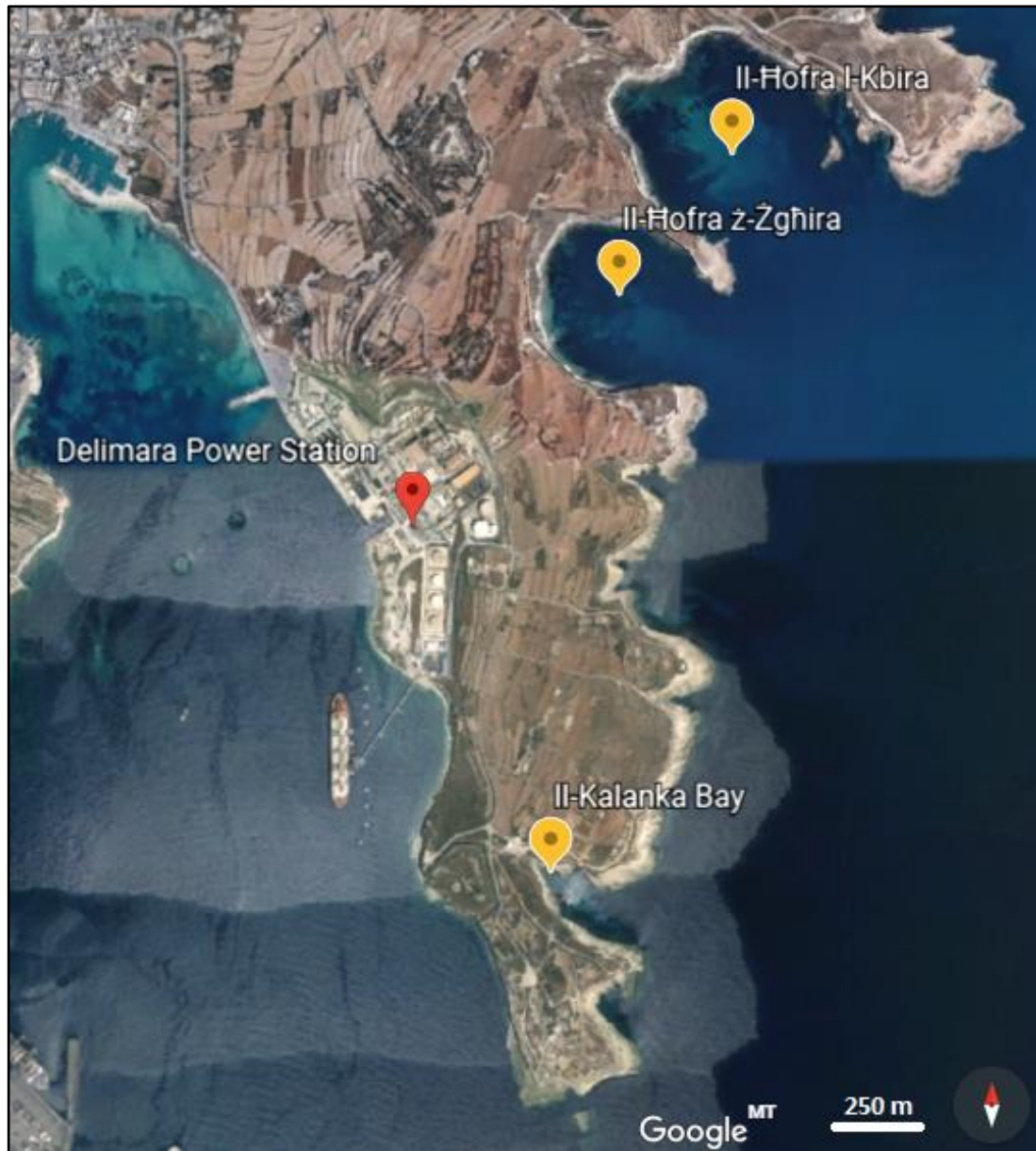
### 2.1 Study sites

Three sites on the southeastern coast of the island of Malta were studied, comprising one directly impacted by the thermal effluent discharged from the Delimara Power Station, and two reference sites. These sites are, respectively: Il-Ħofra ż-Żgħira (henceforth here referred to as ‘impacted site’ or ‘I’); Il-Ħofra l-Kbira (‘reference site 1’ or ‘R1’); and Il-Kalanka it-Tawwalija Bay (‘reference site 2’ or ‘R2’) (Figure 2.1.1).

Sites I and R1 present similar geomorphological characteristics, while R2 is more elongated and narrow and is located further away from the thermal effluent (Figure 2.1.2).



**Figure 2.1.1** – Map showing the location, on the coast of the island of Malta, of the three sites analyzed in this study. Base map from Google Earth Pro 7.3.2.5776, 2019.

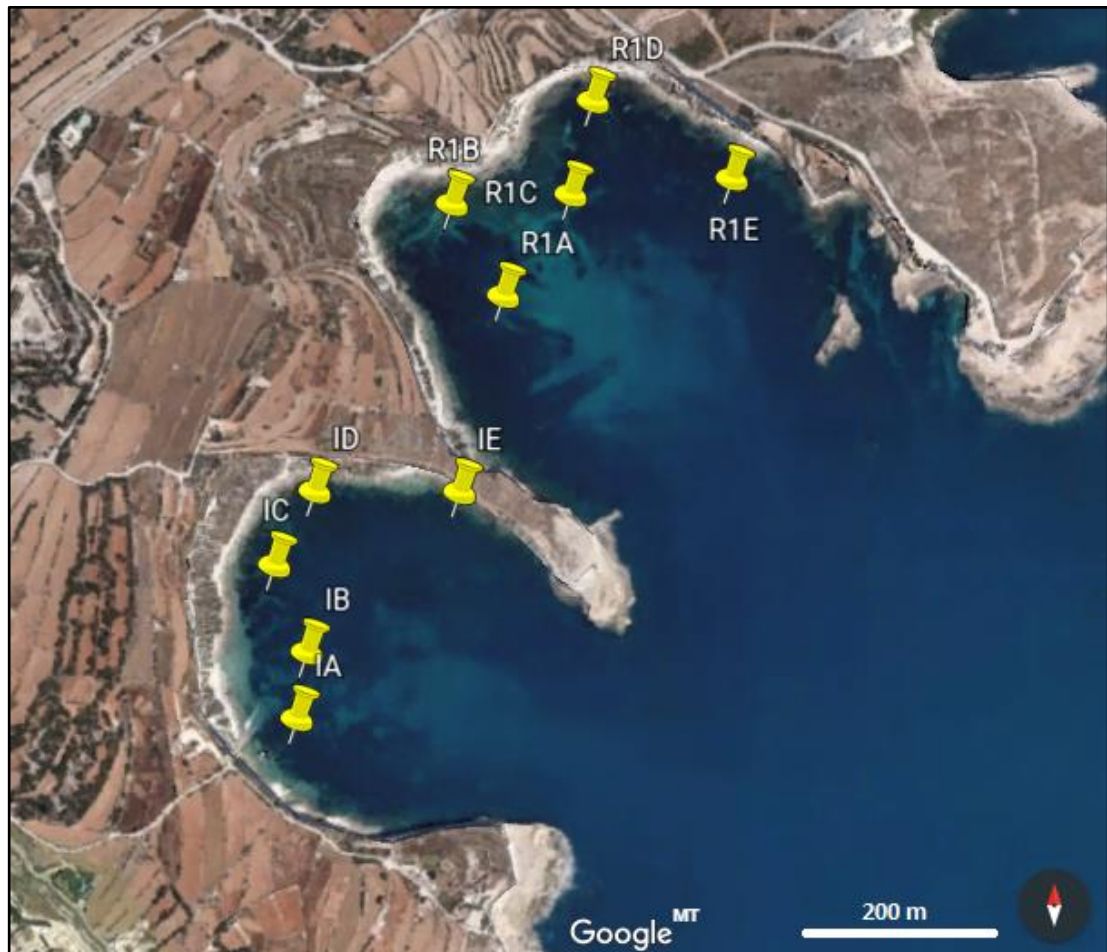


**Figure 2.1.2** – Map showing the geomorphological similarity between sites I (Il-Hofra ż-Żgħira) and R1 (Il-Hofra I-Kbira), as well as the narrow-elongated shape of site R2 (Il-Kalanka Bay). The position of Delimara Power Station between the three bays is marked by the red pin; the thermal effluent from the power station is released into site I. Base map: <https://www.google.com/earth/>, 2019.

## 2.2 Sampling stations

The distribution of sampling stations at site I was based on the water flow pattern generated by the thermal effluent, which creates a current of decreasing temperature that spreads in a clockwise direction within the inlet during both summer and winter (AIS Environmental Ltd., 2011). The same sampling distribution pattern was followed

for the two reference sites. Five stations (A–E) were sampled in each locality, with three replicates collected from each station (Figures 2.2.1 and 2.2.2, replicates not shown). The geographic coordinates of each station, the depth and the mean seawater temperature measured just above the seabed at the time of sampling are summarized in Table 2.2.1; data for other parameters (salinity, conductivity and pH) collected from the sampling stations are given in Appendix A. All fieldwork was conducted in November 2018.



**Figure 2.2.1** – Map showing the distribution of the sampling stations at sites I (stations IA – IE) and R1 (stations R1A – R1E). The source of the thermal effluent outfall position is indicated by the red arrow. Base map from <https://www.google.com/earth/>, 2019.





**Figure 2.2.2** – Map showing the distribution of the sampling stations at site R2. Base map from <https://www.google.com/earth/>, 2019.

**Table 2.2.1** – Geographical coordinates for the sampling stations, the collection depth, and mean temperature (calculated from three readings taken at the time of sampling). Coordinates derived from Google Earth Pro 7.3.2.5776, 2019.

Station	Geographical coordinates		Depth (m)	Mean temperature (°C) ± SD
	Latitude (N)	Longitude (E)		
IA	35°50'09.0"	14°33'37.3"	3.7	22.62 ± 0.02
IB	35°50'11.5"	14°33'37.8"	5.0	23.02 ± 0.08
IC	35°50'14.8"	14°33'36.2"	2.9	22.83 ± 0.01
ID	35°50'17.7"	14°33'38.2"	2.5	22.96 ± 0.01
IE	35°50'17.6"	14°33'45.2"	3.8	22.98 ± 0.03
R1A	35°50'25.0"	14°33'47.0"	4.8	22.67 ± 0.00
R1B	35°50'28.5"	14°33'44.6"	4.3	22.80 ± 0.01
R1C	35°50'29.0"	14°33'50.1"	4.9	22.70 ± 0.01
R1D	35°50'32.4"	14°33'51.2"	2.8	22.83 ± 0.01
R1E	35°50'29.5"	14°33'57.7"	5.2	22.75 ± 0.01
R2A	35°49'24.2"	14°33'37.0"	4.7	22.54 ± 0.01
R2B	35°49'26.0"	14°33'36.0"	3.6	22.61 ± 0.01
R2C	35°49'27.6"	14°33'35.3"	3.0	22.68 ± 0.01
R2D	35°49'26.6"	14°33'38.2"	4.5	22.56 ± 0.00
R2E	35°49'25.9"	14°33'39.5"	6.0	22.55 ± 0.11
Directly at the outfall	35°50'09.2"	14°33'35.7"	–	25.31 ± 0.01

### 2.3 Sampling and staining process

The FOBIMO (FORaminiferal Blo-MONitoring; Schonfeld *et al.*, 2012) protocol was carefully followed for both sampling and staining of the samples. Sediment patches at each location were visually identified and manually sampled. Specimen collection was carried out during autumn (early November, 2018), and it was not compared to the summer population structures due to time and logistical constraints of the present work. However, the FOBIMO Protocol recommends autumn samplings as they offer

the best perennial persistency (Schonfeld *et al.*, 2012). The protocol's recommendation of avoiding reproductive periods was respected.

The surficial sediment layer to a depth of 1 cm was manually sampled until a volume of ca 40 cm<sup>3</sup> was collected for each of the three replicates at each station. The three replicates were collected around 1 m apart from each other, by SCUBA diving in depths between 2.5 m and 6 m. In the laboratory, samples were stained with a solution of Rose Bengal stain in ethanol (2g/L) to differentiate between living specimens at the time of collection (stained) and dead ones (not stained). Samples were gently shaken and, after 14 days in the staining solution, were washed on a 63 µm screen to remove the excess stain; the washed residues were then oven dried at 40°C for 2–3 days to allow dry partitioning.

It is pertinent to mention that the Rose Bengal staining process has limitations. For example, bacteria and organic material attached to the test can be stained, and dead specimens in which the protoplasm still has not completely decayed could also be stained (reviewed by Bernhard, 2000; see also Bernhard, 1988). However, the technique is also argued to be as reliable as others, besides being the most widely used in the scientific community for foraminiferal ecological studies (Murray & Bowser, 2000).

Dead tests could have been brought from other areas and would not faithfully characterize the local abundance, distribution and diversity of foraminiferans in the bays. Therefore, the present study also had the advantage of considering only the stained specimens, differently from what has been done in some studies where a low number of stained specimens led to the analysis of the total foraminiferal assemblage (dead and living; e.g. Caruso & Cosentino 2014a; 2014b).

## **2.4 Subsplitting and laboratory analyses**

A small representative fraction of each sample was analyzed in order to count and identify specimens and, consequently, to assess the absolute and relative abundances of amphisteginids in the total benthic foraminiferal assemblage. For this, the whole sample was initially shaken to mix it well, weighed, and then randomly subsampled using a splitter (Figure 2.4.1) until a portion of between 1–3 g was obtained. From this portion, small quantities of sediment taken at random were spread

on a Petri dish, and foraminiferal individuals were counted and identified. Following the FOBIMO group protocol (Schonfeld *et al.*, 2012), counts were taken until the total target value of at least 300 stained Foraminifera was reached. Tests that showed severe breakage or abrasion were not considered due to the impossibility of identification. Non-amphisteginid benthic Foraminifera in the samples were not identified to species due to time constraints. For each portion or sub-portion analyzed, all of the identifiable specimens of benthic Foraminifera present were manually and individually examined under a stereomicroscope. The specimens were counted, categorized, picked with a wet brush and stored in separate vials according to the following categories:

- Stained *A. lobifera*;
- Non-stained *A. lobifera*;
- Stained *A. lessonii*;
- Non-stained *A. lessonii*;
- Stained non-amphisteginid benthic Foraminifera;
- Non-stained non-amphisteginid benthic Foraminifera;
- Stained *Amphistegina* spp. (specimens too small – possibly juveniles, too abraded, or too deformed to be able to differentiate between *A. lobifera* and *A. lessonii*);
- Non-stained *Amphistegina* spp.

Differentiation between stained and non-stained amphisteginids was based on the colouring of the shell, which shows a white/cream colour when not stained and pink/red when totally or partially (apertural region) stained (see Figure 2 in Guastella *et al.*, 2019). The same colour intensity differences were used for recognizing staining in other species of Foraminifera found in the samples. For assessing absolute and relative abundances, the ratio of *A. lobifera* / *A. lessonii* was taken and the same proportion was assumed to be found for the *Amphistegina* spp. fraction. With that, for the final counts and graphs of total *A. lobifera* abundances, individuals that were not identified to the species level were also considered. Therefore, the total *A. lobifera* counts were considered – i.e. including the extrapolated ratio of *A. lobifera* / *A. lessonii* for the *Amphistegina* spp. fraction.



**Figure 2.4.1** – Photograph showing the splitter device used for unbiased subsampling of the sediment.

## **2.5 Granulometric analysis**

In order to determine the mean sediment grain size and sorting of the samples, a granulometric analysis was carried out by adapting the protocol described by Bale & Kenny (2005).

Differently from the above mentioned protocol, in the present study it was not necessary to initially add sodium hexametaphosphate ( $\text{NaPO}_3$ )<sub>6</sub> to the samples in order to disperse clay particles, since the <63  $\mu\text{m}$  portion had already been removed by sieving during the staining process. Therefore, for each sample, around 25 g of dry sediment was weighed accurately using an electronic balance and transferred to a series of stacked sieves of 4 mm, 2 mm, 1 mm, 500  $\mu\text{m}$ , 250  $\mu\text{m}$ , 125  $\mu\text{m}$  and 63  $\mu\text{m}$  mesh size. The sieves were then mechanically shaken for 15 minutes, at moderate amplitude. The sediment portions retained by each sieve were then weighed individually.



## 2.6 Assessment of the proportion of particles in the samples

In order to estimate the proportion of amphisteginids relative to other particles in the samples, a small fraction (ca 200 grains) of each sediment sample that was retained in the sieves was randomly selected, spread in a Petri dish and then examined under a stereomicroscope. The analyzed fractions were restricted, therefore, to the same size range as for the granulometric analysis (i.e. 4 mm, 2 mm, 1 mm, 500  $\mu\text{m}$ , 250  $\mu\text{m}$ , 125  $\mu\text{m}$  and 63  $\mu\text{m}$ ). The number of amphisteginids and of other particles was counted until approximately a total amount of at least 200 particles was reached. Both stained and non-stained amphisteginids were counted and only the shells in which identification to genus was possible were considered, including therefore slightly broken or abraded specimens that were still identifiable as amphisteginids. Other species of Foraminifera were included in the 'other grains' category. The ratio of amphisteginids to other grains was then extrapolated to the entire weight for each sediment fraction, in order to obtain the total mass of amphisteginids and of other sediment particles in each fraction. For the granulometric analysis, identification to species level was not carried out due to time constraints and because it was not necessary in order to estimate the proportion between the number of particles that were or not amphisteginids (the two species' impacts on sediment granulometrical characteristics are probably similar, and most specimens were *A. lobifera*).

For calculating the extent of change in mean sediment grain size ( $\mu\text{m}$ ) and sorting coefficient ( $\Phi$ ) due to the presence of *Amphistegina* spp., the values for each parameter ( $P$ ) for the sediment without amphisteginids ( $P_S$ ) were compared to sediment with the presence of amphisteginids ( $P_{S+A}$ ) according to the following formula:

$$\% \text{ extent of change per replicate} = \frac{P_{S+A} - P_S}{P_S} \times 100$$

## 2.7 Photographs of *Amphistegina lobifera* for morphological characteristics

As counting and classification of species was executed under the microscope, aberrant morphological abnormalities were individually and qualitatively identified and collected for photographing (see Plate 3.7.2 on Chapter 3). A Nikon SMZ 1500 stereomicroscope with a Nikon DS Ri1 photomicrographic system was used for obtaining the photomicrographs of the specimens at magnifications of 2x or 4x.

Additionally, in order to assess possible morphological deformities that require a more detailed analysis, the stained *A. lobifera* shells from each replicate were randomly subsampled on a splitter (Figure 2.7.1) until around 30 shells were amassed (when this was possible). For the samples that did not contain at least 30 individuals of stained *A. lobifera*, all the available intact shells were photographed. The total number of specimens photographed per station are indicated in Table 2.7.1.

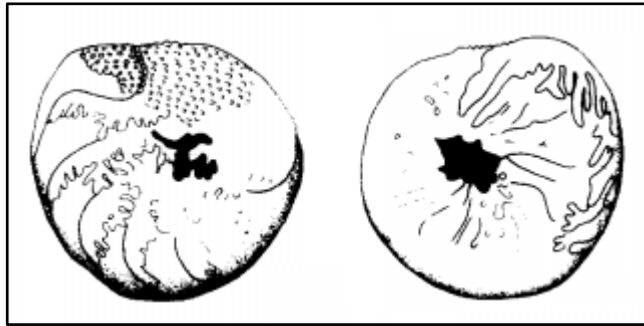
**Table 2.7.1** – Number of photographed specimens per station for assessing greatest spiral diameter (GSD), thickness/diameter ratio (flattening), coiling direction and lateral asymmetry.

Station	Number of specimens	Station	Number of specimens	Station	Number of specimens
IA	93	R1A	91	R2A	75
IB	92	R1B	12	R2B	39
IC	90	R1C	16	R2C	8
ID	90	R1D	33	R2D	91
IE	92	R1E	92	R2E	11

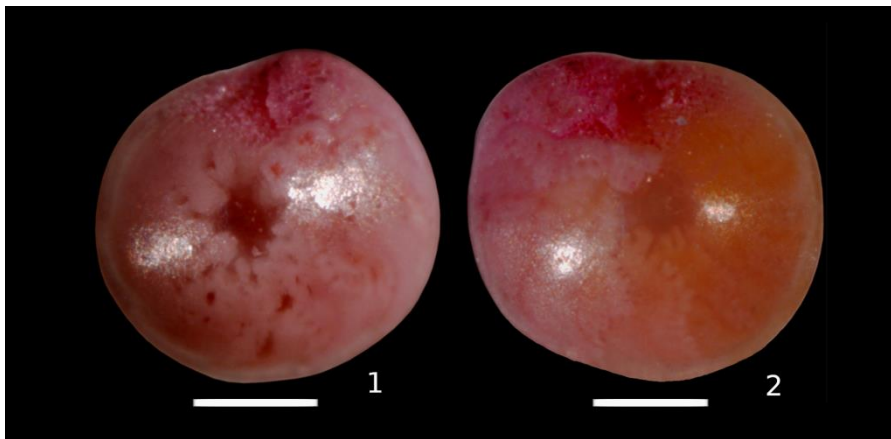


**Figure 2.7.1** – Photograph showing the random subsampling device used for splitting stained *Amphistegina lobifera* shells for photographing.

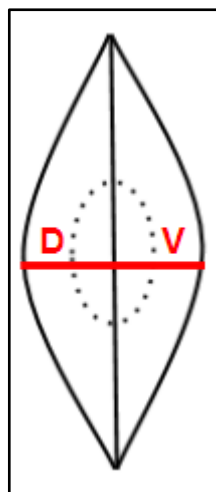
Tests were maintained in position on a Petri dish with the use of a commercial reusable adhesive and both the ventral (umbilical) and lateral (peripheral) view of each test were photographed. The ventral view could be recognized due to the presence and position of the aperture, which also allowed the identification of the coiling direction of the shell. Sinistral specimens have the last chamber present on (or turned to) the left side of the shell when seen in ventral view, the opposite being true for dextral shells (Hallock & Larsen, 1979; Figures 2.7.2 and 2.7.3). Therefore, for the ventral view the specimens were photographed with the aperture placed at the ‘12 o’ clock’ position. For the lateral side, the specimen was then placed with the aperture down, ventral side turned to the right and dorsal side to the left, which led therefore to the lateral view – here considered as the side of the shell opposite to the aperture (Figure 2.7.4).



**Figure 2.7.2** – Diagram of the ventral (left) and dorsal (right) view of a sinistral *Amphistegina lobifera* shell. Modified from Hallock & Larsen, 1979.



**Figure 2.7.3** – Ventral views of a (1) dextral specimen and (2) sinistral specimen of *Amphistegina lobifera* collected in the present study. Scale bars: 0.5mm.



**Figure 2.7.4** – Diagram showing the lateral view as defined in the present study (area opposite to the aperture). The aperture is facing away from the observer and is here represented by the dotted circle; the ventral (umbilical) side of the shell is the one on the right (V), and the dorsal (spiral) side on the left (D).

## 2.8 Shell morphometry

All the photographed specimens were measured with the use of the image analysis software ImageJ v.1.52a (Rasband, 1997–2018). The spiral measurements were made according to Hallock (1979), but in the present study were collected from the umbilical (ventral) side rather than the spiral (dorsal) side as follows: (i) the greatest spiral diameter (GSD, here considered as the largest diameter on the ventral side of the shell); and (ii) the greatest spiral diameter perpendicular to the GSD (SD90) (here also measured on the ventral side). Both measurements are represented in Figure 2.8.1. Additionally, (iii) the lateral diameter, divided into left (dorsal) and right (ventral) portions (see Figure 2.7.4), was also measured following the approach by Harney (1996).

The ratio of thickness to diameter (T/D; flattening) was based on the definition given by Hallock (1979), in which the average spiral diameter refers to:

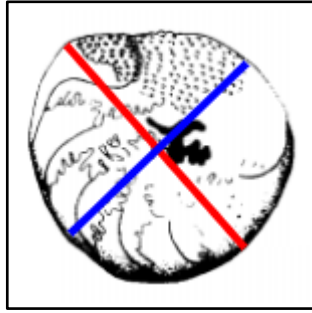
$$\text{average spiral diameter (D)} = (\text{GSD} + \text{SD90}) / 2$$

The final T/D ratio indicates the thickening of the shell, as higher ratios represent thicker shells. Therefore, this ratio was calculated as:

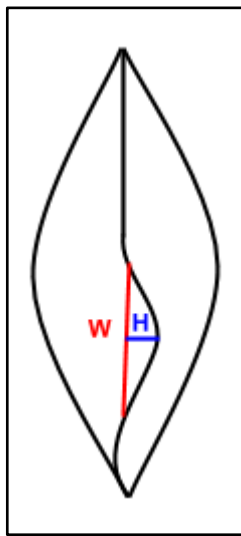
$$\text{thickness/diameter ratio} = \text{total lateral diameter} / \text{average spiral diameter}$$

As stated in section 1.1, there are many different concepts of ‘test thickness’ in the literature. The one used here refers to thickness as being measured from one umbilicus to the other (Hallock & Hansen, 1979; Mateu-Vicens *et al.*, 2009), seen therefore from the lateral view. The lateral asymmetry ratio was calculated as the largest lateral measurement divided by the smallest.

Besides, a classification of different categories of keel/carina shape for *A. lobifera* was created. To facilitate this, the width (W) and height (H) of keel curvature when seen in lateral view were measured using ImageJ (Figure 2.8.2).



**Figure 2.8.1** – Diagram showing the greatest spiral diameter (red line; GSD) and the greatest spiral diameter perpendicular to the greatest spiral diameter (blue line; SD90) measurements as taken in the present study.



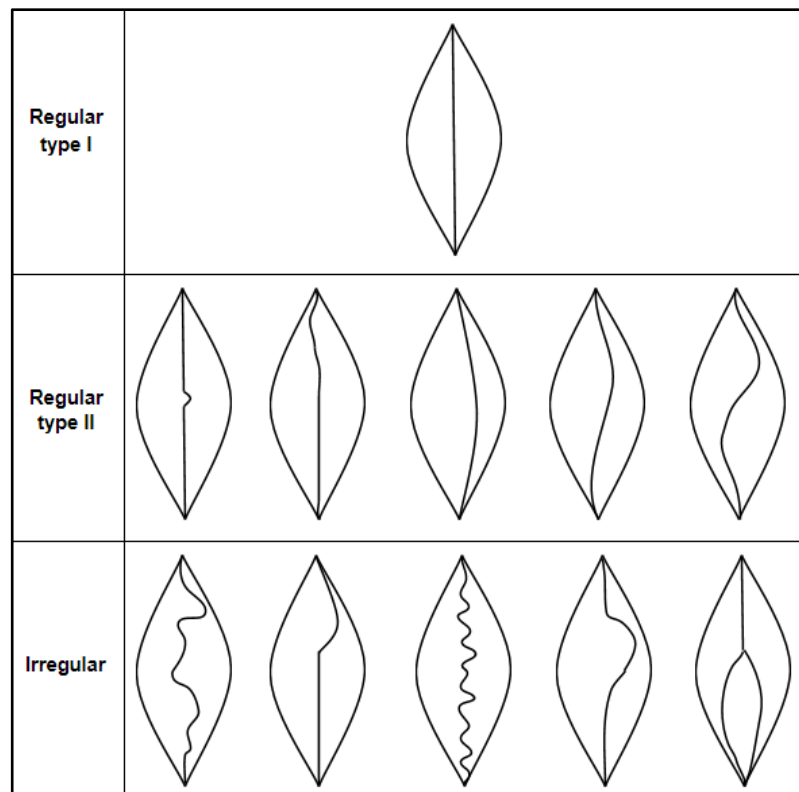
**Figure 2.8.2** – Diagram showing how the measurements to calculate the radius of curvature of the arc representing the sinuous keels of stained *Amphistegina lobifera* were taken. The red line represents the width (W) and the blue line the height (H).

From these measurements, it was possible to calculate the radius of curvature of the arc generated by the curved or sinuous keels using the following formula:

$$R = (((W/2)^2/H)+H)/2$$

The shells were classified into three categories according to the shape of the keel when viewed laterally (Figure 2.8.3), as follows:

- i) regular keel type I, in which the keel took the form of an almost perfectly straight and smooth line and there was no radius of curvature;
- ii) regular keel type II, characterized by a slightly curved or sinuous keel (C-shaped or S-shaped, similar to what has been referred to as “*the wavy keel of the S morphotype*” by Harney, 1996); or, in some cases, by shells showing a more pronounced sinuosity (highly S-shaped) in different nuances, in which the radius of curvature in the keel can approximately vary from 0.08mm to 2.26mm;
- iii) irregular keel, category in which different irregularities were grouped, including abrupt changes in angles or directions, presence of small-scale waviness, bifurcations and other irregularities that therefore prevented the radius of curvature from being calculated.



**Figure 2.8.3** – Diagram showing the main range of variation present in the different categories of regular and irregular keel considered in this study (lateral view of shells).

## 2.9 Data analysis

For statistical analyses, the software Microsoft Office 365 ProPlus – Excel version 1906 was used for creating graphs showing mean values per station for the abundance, granulometric and morphometric parameters. Parametric ANOVA could not be used to test for differences between the three sites since the data did not meet the normal distribution assumption of this test. Instead, permutational multivariate analysis of variance (PERMANOVA) was used to test the null hypothesis of no differences in the analyzed ecological parameters between the impacted and reference sites. The following parameters were tested statistically using this approach: absolute and relative abundance of *A. lobifera*; shell size (greatest spiral diameter), thickness/diameter ratio (flattening) and lateral asymmetry of *A. lobifera*; mean increase in sorting and mean increase in grain size due to the presence of *Amphistegina* spp.

To compare the above mentioned parameters through PERMANOVA, the model was nested and the factor 'Site' was included as fixed, while the factor 'Station' was taken as random and nested within 'Site'. For all analyses, 9999 permutations were applied. For this, the computer software PRIMER v7 for Windows (Clarke & Gorley, 2015) was used. All tests were based on the Euclidean distance measure, making them analogous to traditional ANOVA but with the p-values obtained through permutational analysis. Given the asymmetrical design of the study, with only one impacted site but two reference sites, a contrast was included in the PERMANOVA analyses, in which the impacted site was compared to the two reference sites taken together – (I) vs (R1,R2). The units of replication used were 3 (number of replicate samples) for absolute abundance, relative abundance, % change in grain size and % change in sorting; and up to 32 (number of specimens photographed) for greatest spiral diameter, thickness/diameter ratio (flattening) and lateral asymmetry.

Additionally, for categorical variables (coiling direction and keel shape) expressed as number of individuals per category, a chi-square test was performed using the software PAST v3.25 for Windows (Hammer, Harper & Ryan, 2001) comparing the three sites against each other. Statistical differences were considered as significant for P values lower than 0.05. Granulometric characteristics (mean sediment grain size and sorting), based on the data from the granulometric analysis, were obtained through the software Gradistat v8 (Blott & Pye, 2001).



### 3. Results

#### 3.1 Comparing absolute and relative abundances of *Amphistegina lobifera* between the three sites

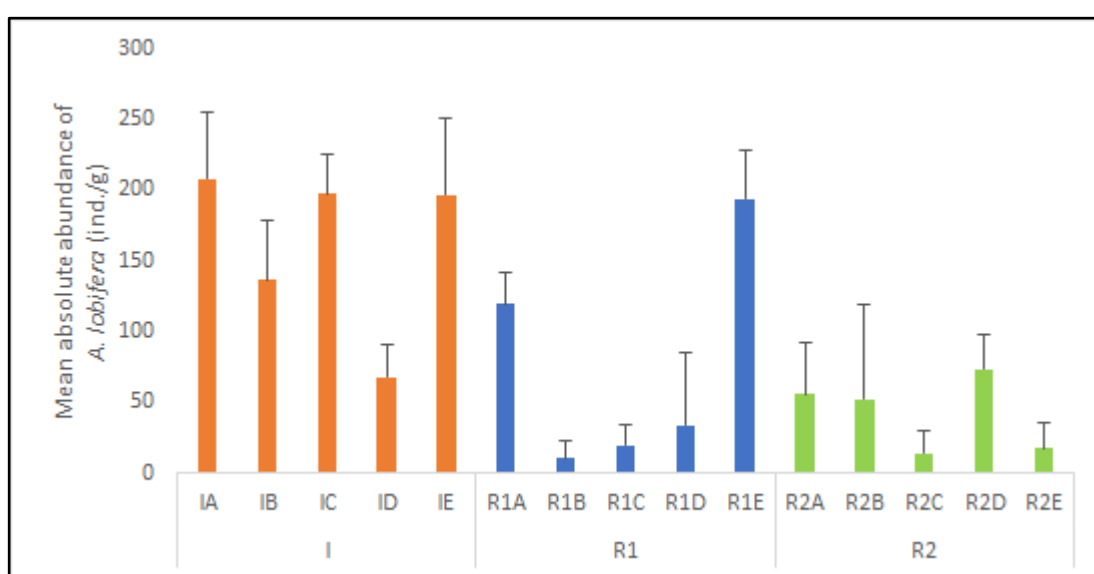
A total of 35,405 individual foraminiferans were recorded in this study, of which 15,631 (44.1%) took up the stain (i.e. were alive at the time of collection) and, therefore, were taken into consideration for the analyses. From the 15,631 stained Foraminifera recorded, about 35% of the specimens (5,568) were found at the impacted site, while 4,999 were recorded at the reference site 1 and 5,064 at the reference site 2. The total absolute abundance counts (number of individuals per gram of analyzed sediment) for the different categories of Foraminifera per site are shown in Table 3.1.1, while the entire abundance count dataset is given in Tables B1 – B.4, Appendix B.

**Table 3.1.1** – Total absolute abundance of Foraminifera (individuals/g) for each site. [I = impacted site; R1 = reference site 1; R2 = reference site 2]

Analyzed categories	I (ind./g)	R1 (ind./g)	R2 (ind./g)
Stained <i>Amphistegina lobifera</i>	127	77	43
Non-stained <i>Amphistegina lobifera</i>	1	0	0
Stained <i>Amphistegina lessonii</i>	6	12	17
Non-stained <i>Amphistegina lessonii</i>	0	0	3
Stained <i>Amphistegina</i> spp.	21	18	16
Non-stained <i>Amphistegina</i> spp.	1	0	3
Stained non-amphisteginid benthic Foraminifera	62	310	354
Non-stained non-amphisteginid benthic Foraminifera	213	566	626
Total stained Foraminifera	216	418	429
Total analyzed sediment weight (g)	26	12	12

The absolute abundance of *A. lobifera* in the impacted site was of ca 161 individuals/g, against around 75 ind./g in the reference site 1 and 43 ind./g in reference 2.

Considering the total *A. lobifera* counts (which include the extrapolated values from the *Amphistegina* spp. fraction), the impacted site presented an overall higher absolute abundance of *A. lobifera* (up to around 200 ind./g) when compared to the two reference sites (Figure 3.1.1). Nevertheless, station ID showed a lower absolute abundance than the other impacted stations, while reference station R1E exhibited as many *A. lobifera* specimens as most of the impacted stations. Of the three sites, reference site 2 presented the lowest overall absolute abundance levels. PERMANOVA indicated a significant difference between the absolute abundances of *A. lobifera* in the impacted site when contrasted to the two reference sites (Table 3.1.2).

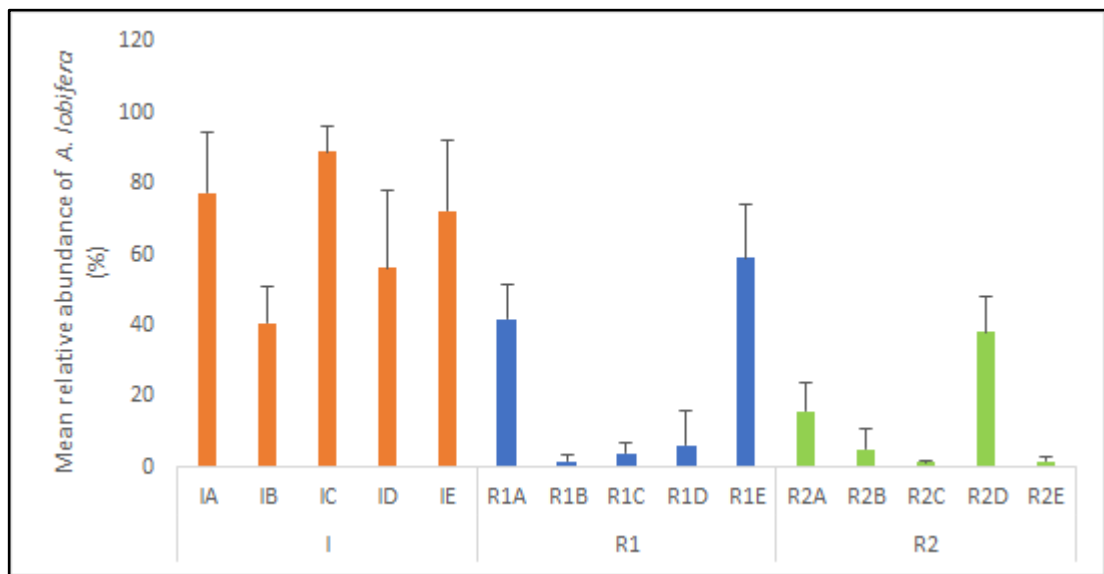


**Figure 3.1.1** – Mean absolute abundance of *Amphistegina lobifera* (ind./g) per station in the three studied sites. Error bars represent +SD. [I = impacted site; R1 = reference site 1; R2 = reference site 2].

**Table 3.1.2** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in absolute abundance of *Amphistegina lobifera* between the impacted and reference sites.

Source of variation	df	Sum of squares (SS)	Mean of squares (MS)	Pseudo-F	P (perm)
Site	2	.12E+05	56049	5.3863	0.0217
Contrast: impacted site vs reference sites	1	1.04E+05	1.04E+05	10.199	0.0059
Residuals	30	39833	1327.8	–	–

With regards to the relative abundance of *A. lobifera* (%) when compared to the total stained foraminiferal assemblage, similar overall patterns as seen for absolute abundance were observed (Figure 3.1.2). The impacted site presented the highest mean relative abundance of the species (ca 67%) when compared to the reference sites (around 22.5% in R1 and 12.4% in R2; Table B.5, Appendix B). However, both for absolute and relative abundance some stations in the reference sites (i.e. R1A, R1E and R2D) showed higher values of *A. lobifera* than the other stations from the same sites.



**Figure 3.1.2** – Mean relative abundance of *Amphistegina lobifera* (%) per station in the three studied sites. Error bars represent +SD.

The PERMANOVA analysis indicated a significant difference in the relative abundance of *A. lobifera* between the impacted and reference sites (Table 3.1.3). The complete results for all the statistical analysis performed in the present study are compiled in Appendix C.

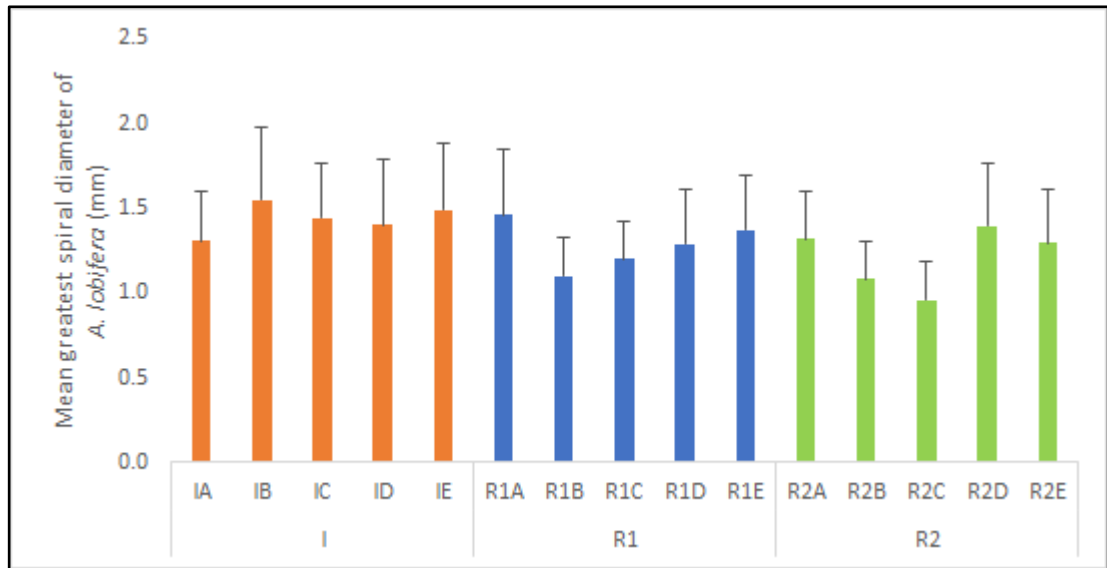
**Table 3.1.3** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in relative abundance of *Amphistegina lobifera* between the impacted and reference sites.

Source of variation	df	Sum of squares (SS)	Mean of squares (MS)	Pseudo-F	P (perm)
Site	2	25298	12649	9.8673	0.0063
Contrast: impacted site vs reference sites	1	24527	24527	19.738	0.002
Residuals	30	3944.8	131.49	–	–

### 3.2 Greatest spiral diameter (GSD)

A total of 924 shells were analyzed for assessing mean size in the studied populations. The overall mean GSD (mm) for the analyzed *A. lobifera* tests is shown in Figure 3.2.1. The number of specimens photographed for measuring GSD and other morphological parameters per replicate sample, and within each site and station, are compiled in Table B.6, Appendix B.

Tests collected in this study varied in mean size between approximately 1.0mm and 1.5mm. The highest GSD value found was 2.877mm (station IB), and the lowest was 0.405mm (station R1A). Overall, the impacted site presented tests with slightly larger sizes than the reference sites. Reference site 2 showed the overall smallest test sizes. PERMANOVA demonstrated a significant difference in test sizes between the impacted and reference sites (Table 3.2.1).



**Figure 3.2.1** – Mean values of greatest spiral diameter (mm) of *Amphistegina lobifera* per station in the three studied sites. Error bars represent +SD.

**Table 3.2.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in greatest spiral diameter of *Amphistegina lobifera* between the impacted and reference sites.

Source of variation	df	Sum of squares (SS)	Mean of squares (MS)	Pseudo-F	P (perm)
Site	2	5.265	2.6325	5.4098	0.0298
Contrast: impacted site vs reference sites	1	5.2122	5.2122	8.8944	0.0126
Residuals	909	110.32	0.12137	–	–

### 3.3 Incidence of irregular keel

Out of the entire sample of 924 shells used for morphological investigations, 918 shells were analyzed for assessing the keel shape (incidence of irregular keel); it was not possible to assess keel shape for the other 6 specimens. The total counts of specimens with regular and irregular keel types are shown in Table 3.3.1. Irregular keels were significantly more common at the impacted site (35%) compared to the two reference sites (25–28%). Chi-square analysis indicated that the incidence of irregular keels varied significantly between the three sites (Table 3.3.2). Pairwise chi-square tests showed no difference in the incidence of irregular keels between the two reference sites, while a significant difference was found when comparing the impacted

site with reference site 2. In the case of sites I and R1, the recorded p-value ( $p=0.0678$ , see Table 3.3.2) was only marginally higher than the level of significance ( $\alpha = 0.05$ ), so the difference in the incidence of irregular keel between these two sites can be considered as almost statistically significant.

**Table 3.3.1** – Counts and percentage abundance of regular and irregular keel morphologies in *Amphistegina lobifera* at the three sites.

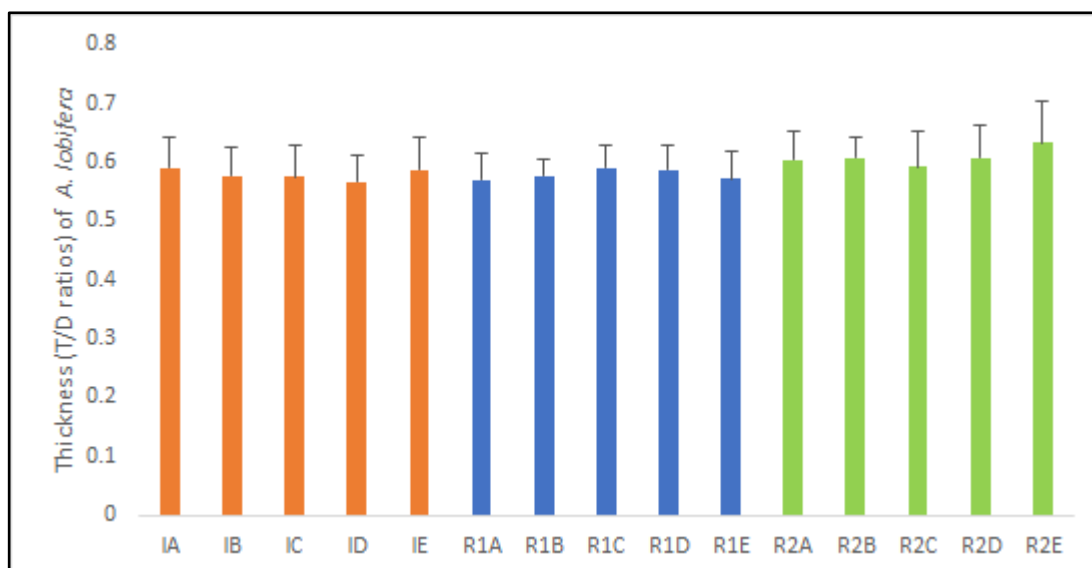
	I	R1	R2
Regular keel	295	171	166
Irregular keel	161	68	57
Total	456	239	223
% Regular	64.69%	71.55%	74.44%
% Irregular	35.31%	28.45%	25.56%

**Table 3.3.2** – Chi-square values of the counts of incidence of irregular keel in *Amphistegina lobifera* in the three sites.

Values tested	Chi-square	P	df
I vs R1 vs R2	7.7333	0.0209	2
I vs R1	3.3355	0.0678	1
I vs R2	6.5265	0.0106	1
R1 vs R2	0.4887	0.4845	1

### 3.4 Thickness/diameter (T/D) ratio

The results for mean T/D ratio (flattening) per station are shown in Figure 3.4.1, and the entire dataset is presented in Table B.7, Appendix B. For the 924 shells analyzed, there were no significant differences found in the T/D ratios between the three sites (Table 3.4.1). The highest ratio found was of 0.852 (station IE), and the lowest was 0.449 (station R1A). The mean T/D ratio for all the stations from all the sites was of 0.585, which is similar to the means for the individual stations. Therefore, the three sites presented shells of similar flattened morphology with a diameter that was overall around twice the shell thickness.



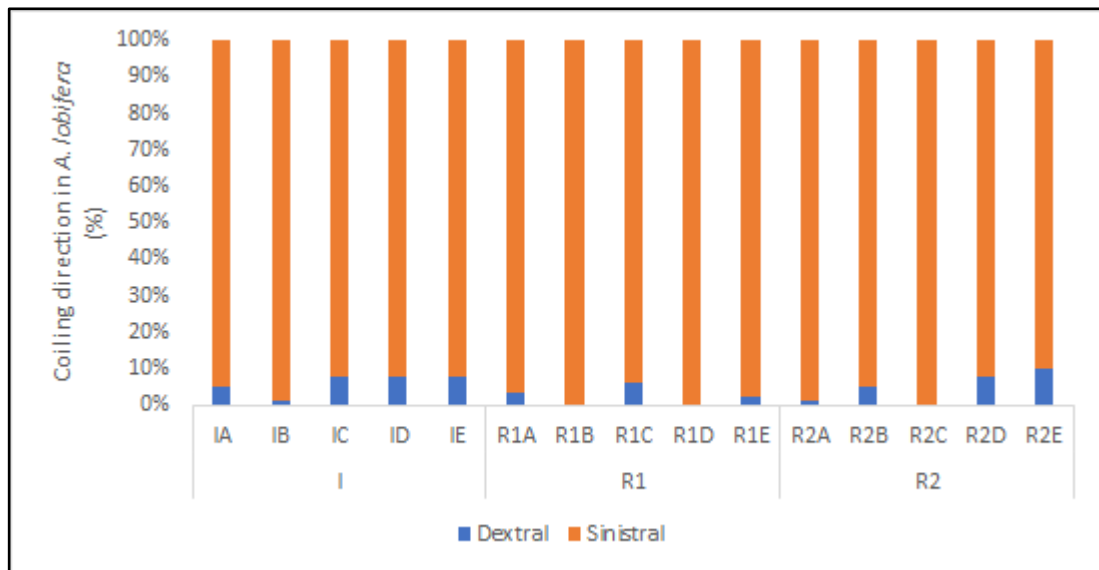
**Figure 3.4.1** – Mean values of thickness/diameter ratio of *Amphistegina lobifera* per station in the three studied sites. Error bars represent +SD.

**Table 3.4.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in thickness/diameter ratio of *Amphistegina lobifera* between the impacted and reference sites.

Source of variation	df	Sum of squares (SS)	Mean of squares (MS)	Pseudo-F	P (perm)
Site	2	0.0392	0.0196	1.9683	0.2005
Contrast: impacted site vs reference sites	1	0.0069	0.0069	0.5218	0.4936
Residuals	909	10.814	0.0119	–	–

### 3.5 Coiling direction

Of the 924 tests of *A. lobifera* studied, 95.24% were sinistral. For all the stations, the same pattern of at least 90% of individuals being sinistral was found (Figure 3.5.1; see Table B.8 in Appendix B for entire coiling direction dataset). The lowest percentage of sinistral specimens was of 90% (station R2E), and the highest was of 100% sinistral specimens (stations R1B, R1D and R2C). No statistically significant difference was found in the occurrence of dextral/sinistral specimens between the three sites (Table 3.5.1). Therefore, temperature did not appear to have any significant influence on the coiling direction of *A. lobifera* in the present study.



**Figure 3.5.1** – Proportion (%) of coiling direction (dextral/sinistral) in *Amphistegina lobifera* per station.

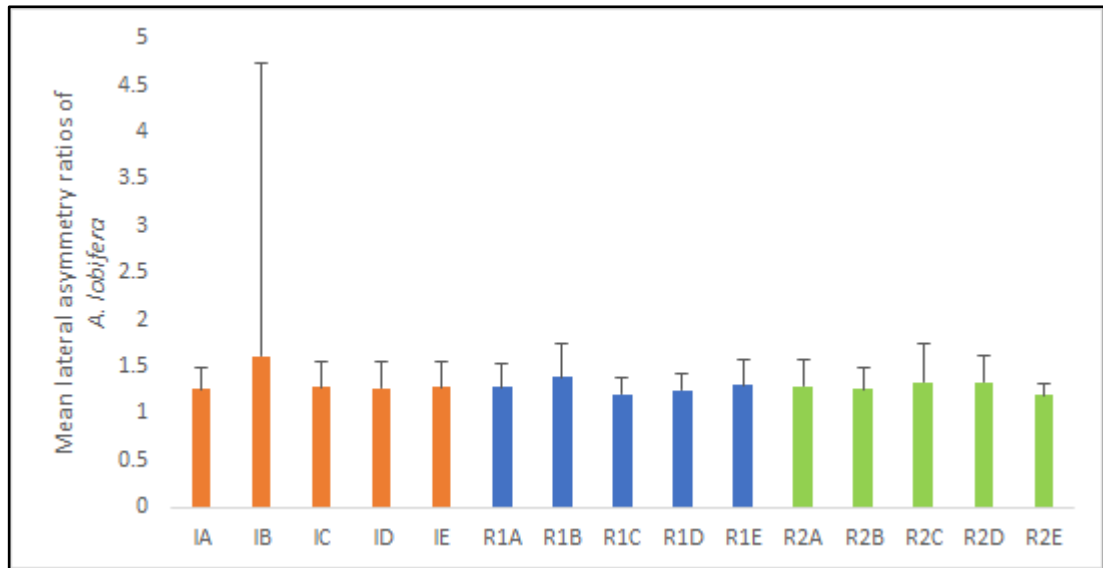
**Table 3.5.1** – Chi-square values of the counts of incidence of dextral/sinistral specimens of *Amphistegina lobifera* in the three sites.

Values tested	Chi-square	P	df
I vs R1 vs R1	4.1915	0.1230	1

### 3.6 Lateral asymmetry

The mean lateral asymmetry ratio for each site and per station are displayed in Figure 3.6.1 (entire dataset in Table B.9, Appendix B). Overall, there were no significant differences found when analyzing 924 shells (Table 3.6.1). However, when analyzing the mean lateral asymmetry ratios of *A. lobifera*, a slightly higher value for station IB was noted, as well as a clearly higher standard deviation value for this station. Such discrepancy is due to the presence of two outliers that showed a highly asymmetrical test having a ‘D shape’ which, therefore, influenced the mean value for this station. The outlier tests are shown in Figure 3.6.2, along with one example of a specimen that is slightly asymmetrical and one example of a symmetrical test. The mean lateral asymmetry ratios ranged between around 1.20 and 1.39 (Table 3.6.2). With the exception of one of the outliers found in the impacted site, which showed a lateral asymmetry ratio of 31.042, the highest value of lateral asymmetry ratio found was of 2.756 (value found in station IE). The lowest value was 1.000, indicating a symmetrical shell (value recorded from individuals found in all three sites).





**Figure 3.6.1** – Mean values of lateral asymmetry ratios of *Amphistegina lobifera* per station in the three studied sites. Error bars represent +SD.

**Table 3.6.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in lateral asymmetry ratios in *Amphistegina lobifera* between the impacted and reference sites.

Source of variation	Df	Sum of squares (SS)	Mean of squares (MS)	Pseudo- <i>F</i>	<i>P</i> (perm)
Site	2	0.4409	0.2204	0.2551	0.8832
Contrast: impacted site vs reference sites	1	0.4323	0.4328	0.5599	0.5518
Residuals	909	948.66	1.0436	–	–



**Figure 3.6.2** – Photomicrographs of the lateral views of *Amphistegina lobifera* tests showing the two outlier specimens presenting a ‘D shape’ (1a and 1b), a specimen that is slightly asymmetrical (2) and a symmetrical one (3). Scale bar: 0.5mm.

### 3.7 Aberrant morphologies

The normal general morphological characteristics of *A. lobifera*, especially with regards to its smooth and round shape (Figure 3.7.1), present some variation as is to be expected in natural populations. However, in this study the presence of severely aberrant morphologies was noted. These deformities were qualitatively identified in the shells analyzed for counting and photographing, which were then categorized into different types. The above mentioned approach of considering only the severe morphological abnormalities due to the presence of a natural range of phenotypes in populations was also taken by Alve (1991).

In total, 33 aberrant individuals were recorded out of 4,702 *A. lobifera* shells (Table 3.7.1). The deformities were only considered when present in shells in which the identification of the species was possible, and therefore *Amphistegina* spp. specimens were not taken into consideration. Description of the deformities and the sites of occurrence are shown in Table 3.7.2. In total, 9 types of deformities were recorded, which are summarized in Figure 3.7.2 with photomicrographs of representative examples.

Five out of the nine deformities were found in both impacted and reference sites (i.e. both in the impacted and in at least one of the reference sites). The only types of

deformities to be found exclusively in a specific site were: i) restricted to the impacted site – shell contour interrupted by a flat area (specimen 9 in Figure 3.7.2), shell with an irregular protuberance/outline (specimen 10) and shell with double aperture (specimen 14); and ii) restricted to R2 – elongated shell with waviness and a small protuberance (specimen 13).



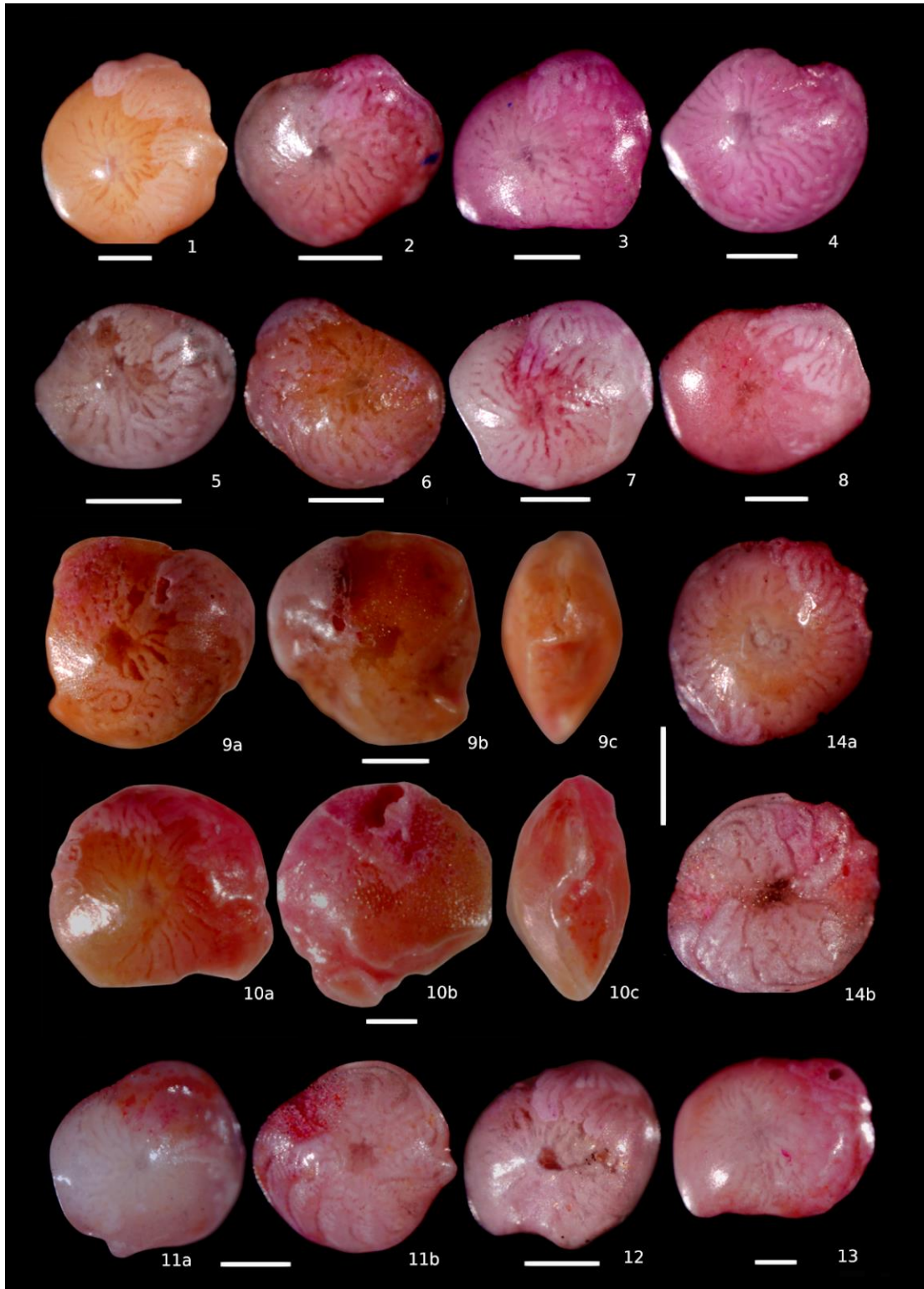
**Figure 3.7.1** – Photomicrograph of the dorsal view of a stained, non-deformed specimen of *Amphistegina lobifera* with typical morphology. Scale bar: 0.5mm.

**Table 3.7.1** – The number of severely aberrant *Amphistegina lobifera* at the investigated sites.

Site	Number of severely deformed individuals	Total number of <i>Amphistegina lobifera</i> specimens	Extent of severely aberrant morphologies in the population
Impacted	19	3275	0.58%
Reference 1	9	925	0.97%
Reference 2	5	502	0.99%
Total	33	4702	0.70%

**Table 3.7.2** – Description of the different types of severely aberrant morphologies encountered during the present study, and the sites where they occurred. Specimen numbers refer to the examples illustrated in Figure 3.7.2.

<b>Deformity</b>	<b>I</b>	<b>R1</b>	<b>R2</b>
Presence of waviness in the test outline (specimens 1 and 2, dorsal views).	x	x	
Presence of flattening on only one side of the test (specimens 3 and 4, dorsal views).	x	x	x
Elongated test shape (specimens 5 and 6, dorsal views).	x	x	x
Two or more abrupt changes in angles (many flat areas), generating a 'squarish' shape (specimens 7 and 8, dorsal views).	x	x	x
The contour of the shell is interrupted by a flat area (specimen 9 – 9a dorsal view, 9b ventral and 9c lateral view).	x		
Irregular protuberance and outline (specimen 10 – 10a dorsal view, 10b ventral and 10c lateral view ).	x		
Presence of one or more regular protuberances (specimens 11 and 12 – 11a and 12 dorsal views, 11b lateral view).	x	x	
Elongated shell with waviness and a small protuberance (three features together; specimen 13, dorsal view).			x
Individual with a double aperture and consequently double umbilicus, with the ventral side strongly curved back in a V-shape and a connection between the two apertures due to the presence of pustules in the central area of the umbilical (ventral) side (specimen 14 – 14a dorsal view, 14b ventral).	x		



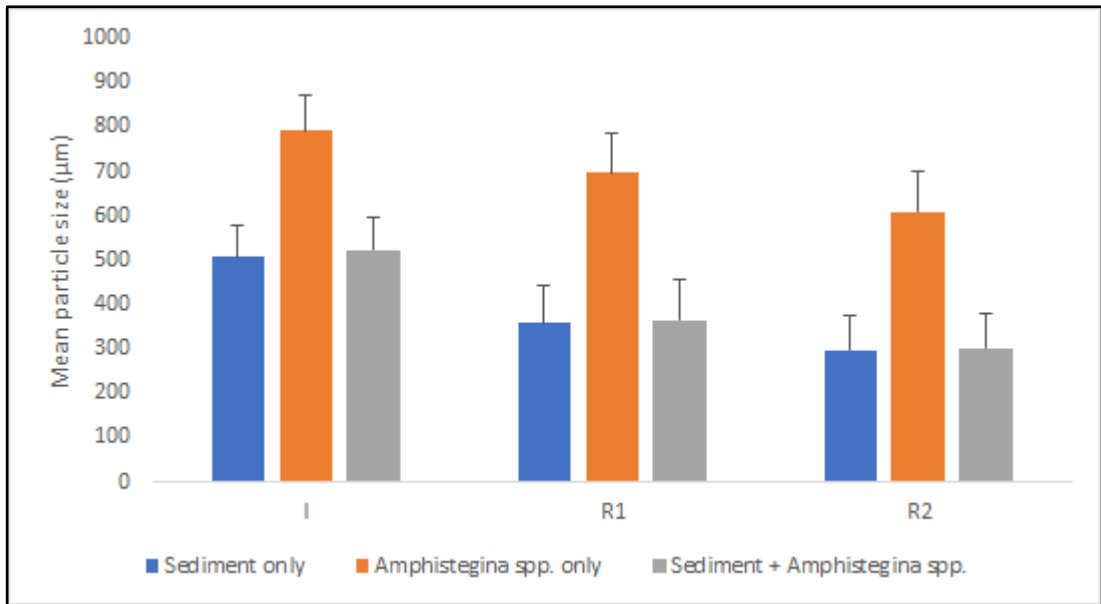
**Figure 3.7.2** – Photomicrographs of Rose Bengal stained *Amphistegina lobifera* specimens found in the studied sites which show severe morphological deformities. The deformities are described in Table 3.7.2. Scale bars: 0.5mm.

### 3.8 Mean particle size

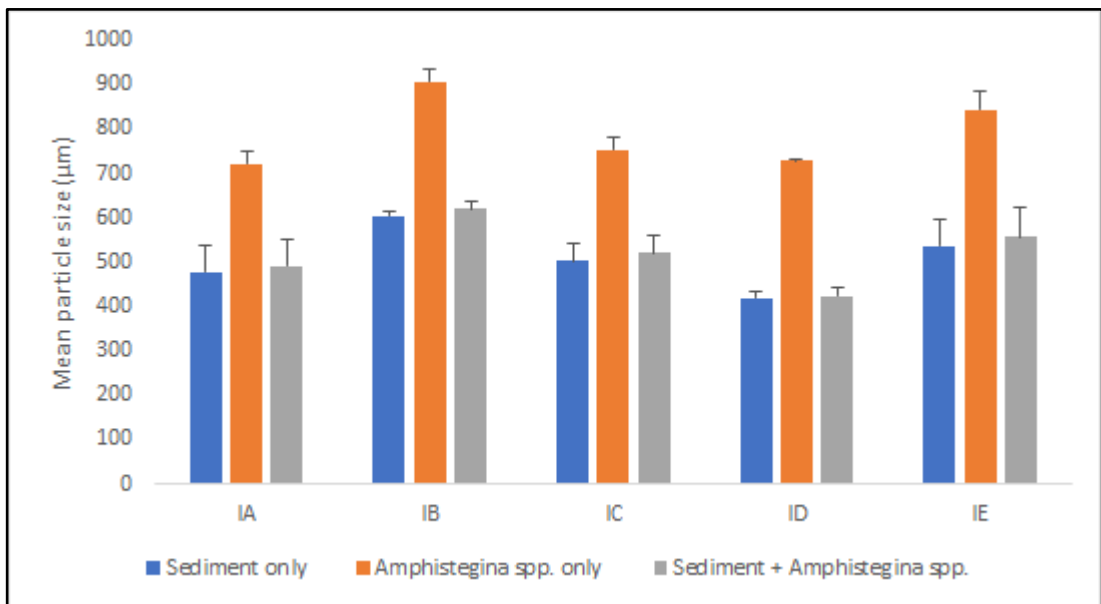
The mean particle size ( $\mu\text{m}$ ) of the sediment at the analyzed sites and stations is summarized in Figures 3.8.1 – 3.8.4, and the mean percentage change in sediment grain size due to the presence of amphisteginids for each station is shown in Figure 3.8.5. The particles measured in the present study refer to sediment without amphisteginids ('sediment only'), amphisteginids ('*Amphistegina* spp. only') and the total sediment including amphisteginids ('sediment + *Amphistegina* spp.'). The full mean particle size dataset is given in Table B.10, Appendix B and the granulometric analyses results are given in Appendix D.

For all the stations, the mean size of amphisteginid tests was always larger than that of the other sediment particles and also than that of the sediment plus amphisteginids. The mean grain sizes of the sediment with and without amphisteginids were similar for all stations in the three sites. However, when comparing the extent of change (%) in mean sediment grain size due to the presence of amphisteginids between the three sites, a significant difference was found between the impacted and the reference sites (Table 3.8.1). Nevertheless, it is notable that these percentage differences are small (up to around 4%).

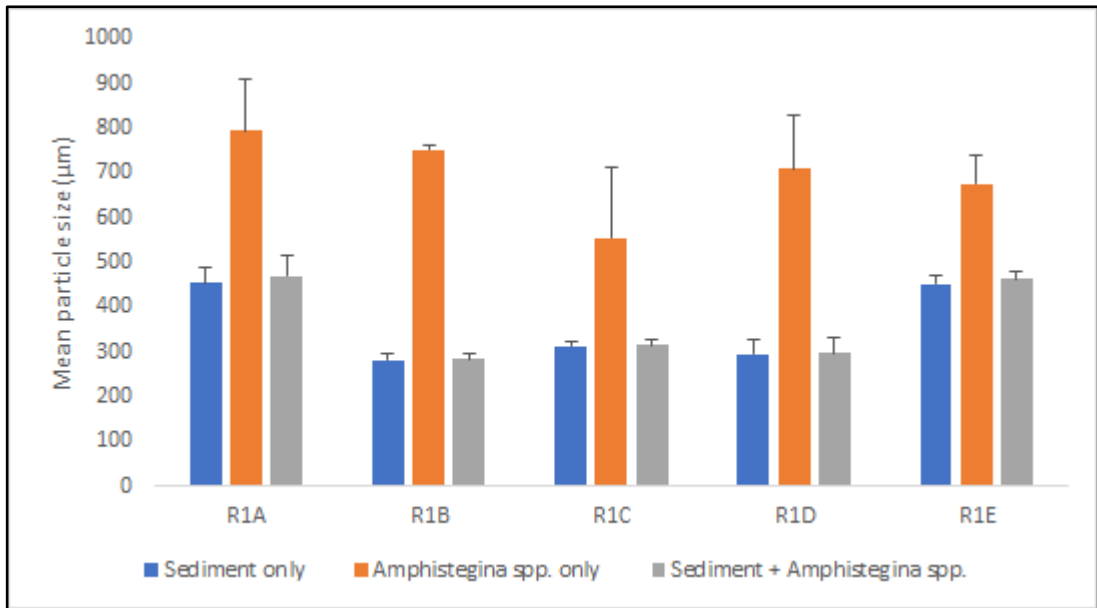
The largest mean sediment grain size occurred in station IB (ca 620.52  $\mu\text{m}$ ), and the lowest in station R2E (ca 211.16  $\mu\text{m}$ ). According to the Udden-Wentworth Grain-Size Scale (Udden, 1914; Wentworth, 1922), most of the stations are classified as 'medium sand' while all those classified as 'coarse sand' occurred in the impacted site (Figure 3.8.1). When compared to the reference sites, the impacted site had the coarsest sediment both with and without amphisteginids (Figure 3.8.1). However, even though the mean size of amphisteginids tests was larger at the impacted site, it was still similar to the the means found in the reference sites.



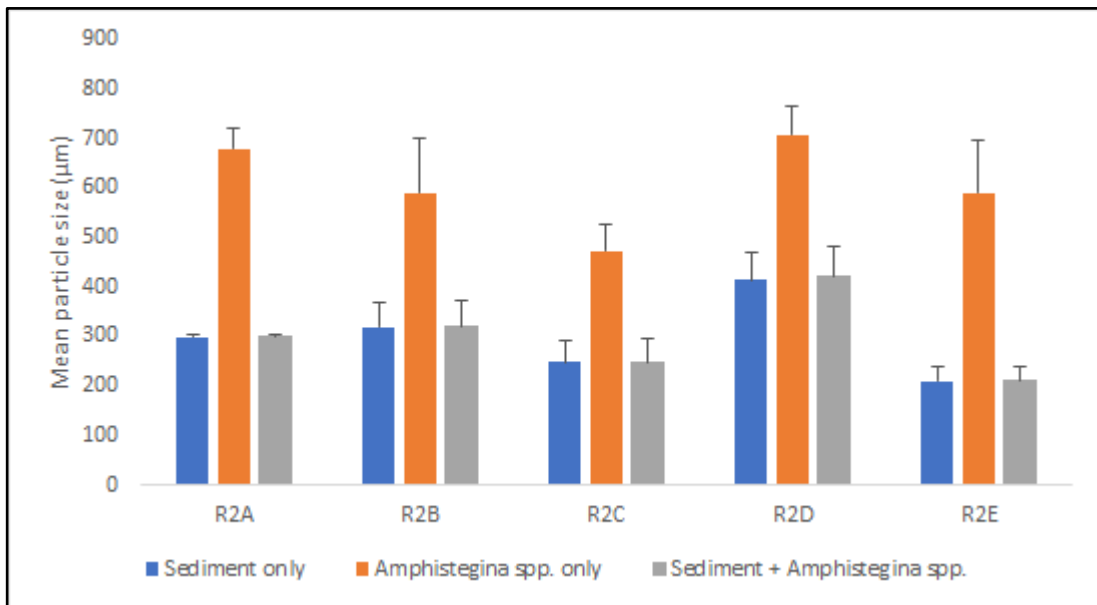
**Figure 3.8.1** – Mean particle size ( $\mu\text{m}$ ) of the different categories analyzed per site. Error bars represent +SD.



**Figure 3.8.2** – Mean particle size ( $\mu\text{m}$ ) of the different categories analyzed per station in the impacted site. Error bars represent +SD.

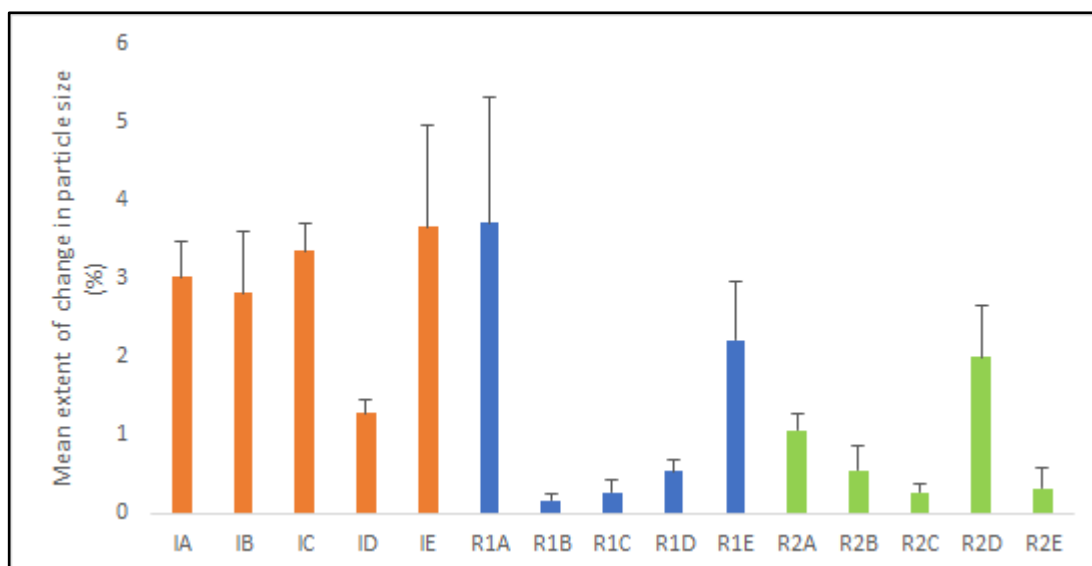


**Figure 3.8.3** – Mean particle size ( $\mu\text{m}$ ) of the different categories analyzed per station in the reference site 1. Error bars represent +SD.



**Figure 3.8.4** – Mean particle size ( $\mu\text{m}$ ) of the different categories analyzed per station in the reference site 2. Error bars represent +SD.





**Figure 3.8.5** – Mean extent of change in particle size (%) due to the presence of *Amphistegina lobifera* per station in the three analyzed sites. Error bars represent +SD.

**Table 3.8.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in the extent of change (%) in mean sediment grain size due to the presence of *Amphistegina lobifera* between the impacted and reference sites.

Source of variation	df	Sum of squares (SS)	Mean of squares (MS)	Pseudo- <i>F</i>	<i>P</i> (perm)
Site	2	31.634	15.817	4.2099	0.0495
Contrast: impacted site vs reference sites	1	29.438	29.438	8.0939	0.0184
Residuals	30	13.019	0.4340	–	–

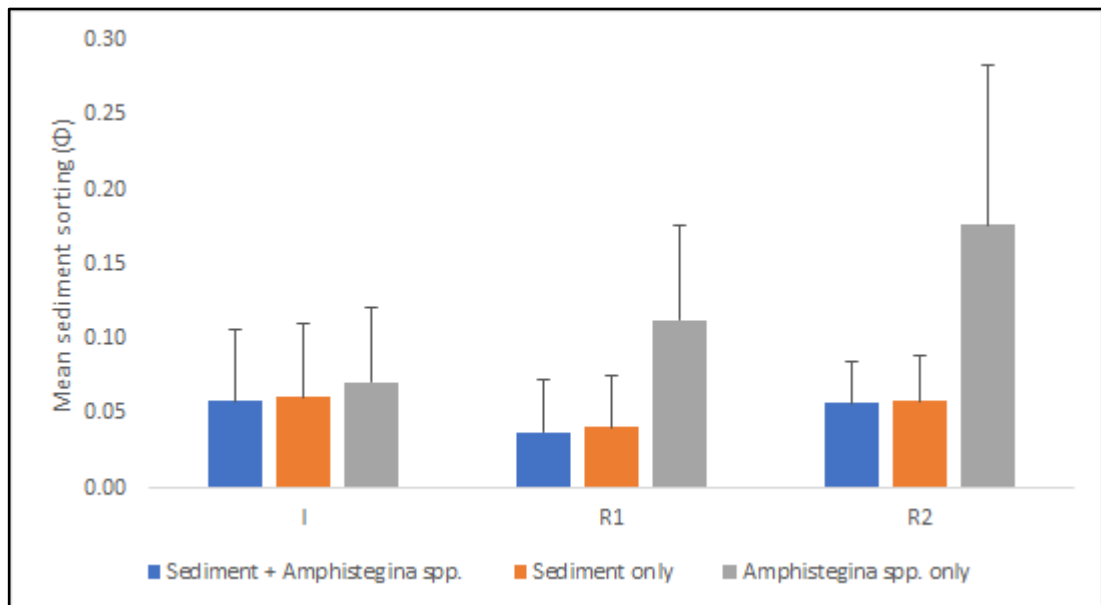
### 3.9 Particle sorting

The mean sorting coefficient value ( $\Phi$ ) for each site and station is illustrated in Figures 3.9.1 – 3.9.4 (entire sorting dataset available in Table B.11, Appendix B). As for mean particle size (section 3.8), sorting was assessed for ‘sediment only’, ‘*Amphistegina* spp. only’ and ‘sediment + *Amphistegina* spp.’.

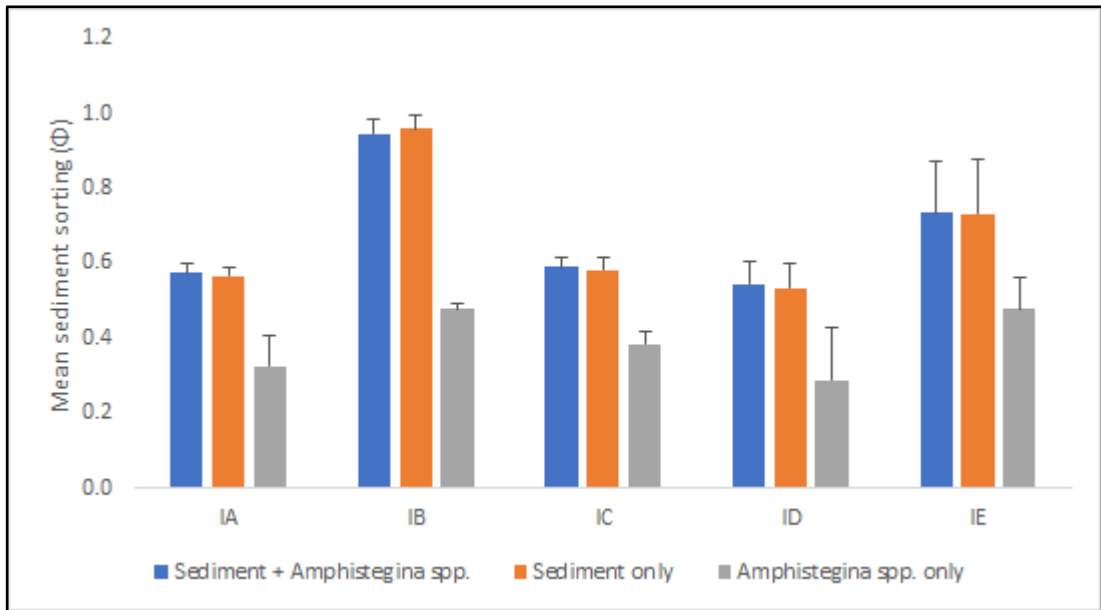
For the three sites, no significant difference was found in sediment sorting with and without amphisteginids (Figure 3.9.1). Nevertheless, once again in spite of the lack of a statistically significant difference and of the differences in sorting values between

the sites being minor, the sorting values of *Amphistegina* spp. *per se* are slightly higher in the reference sites than in the impacted site. This would mean that most amphisteginids found at the impacted site had similar-sized tests, whereas a greater variation in test sizes was present at the reference sites, resulting in a higher sorting value (i.e. more poorly sorted particles) for the latter.

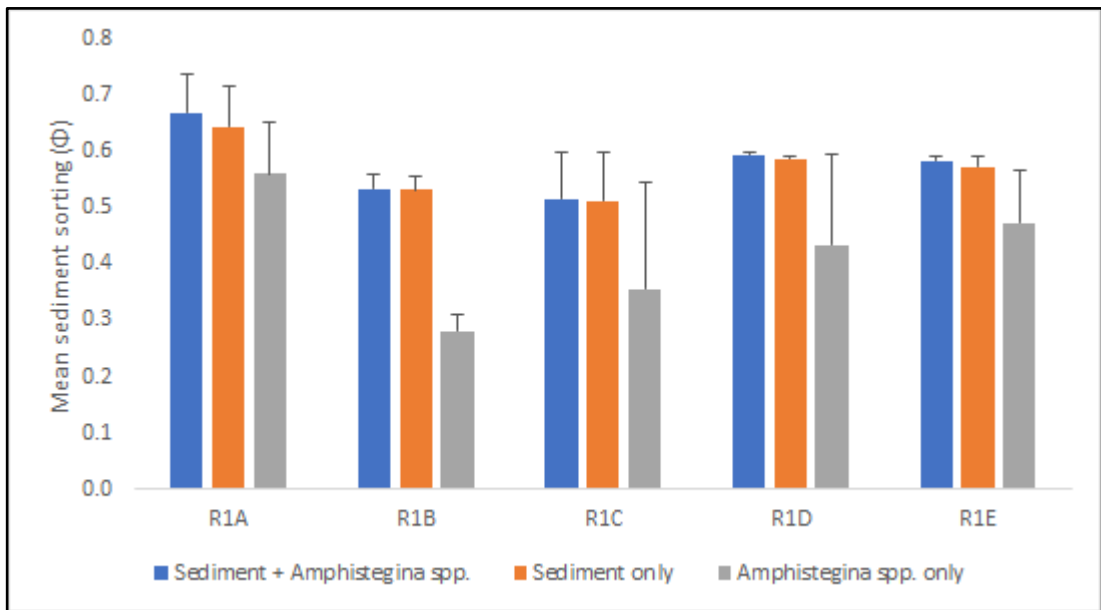
Following the Udden-Wentworth Scale classification, the impacted site and reference site 2 presented stations with sediment varying from very well sorted to moderately sorted (sorting coefficients –  $\Phi$  – from 0.202 to 1.000 in I, and from 0.000 to 0.777 in R2), while R1 showed stations classified as from very well sorted to moderately well sorted ( $\Phi$  values of 0.258 – 0.730). Therefore, none of the analyzed stations was classified as poorly sorted.



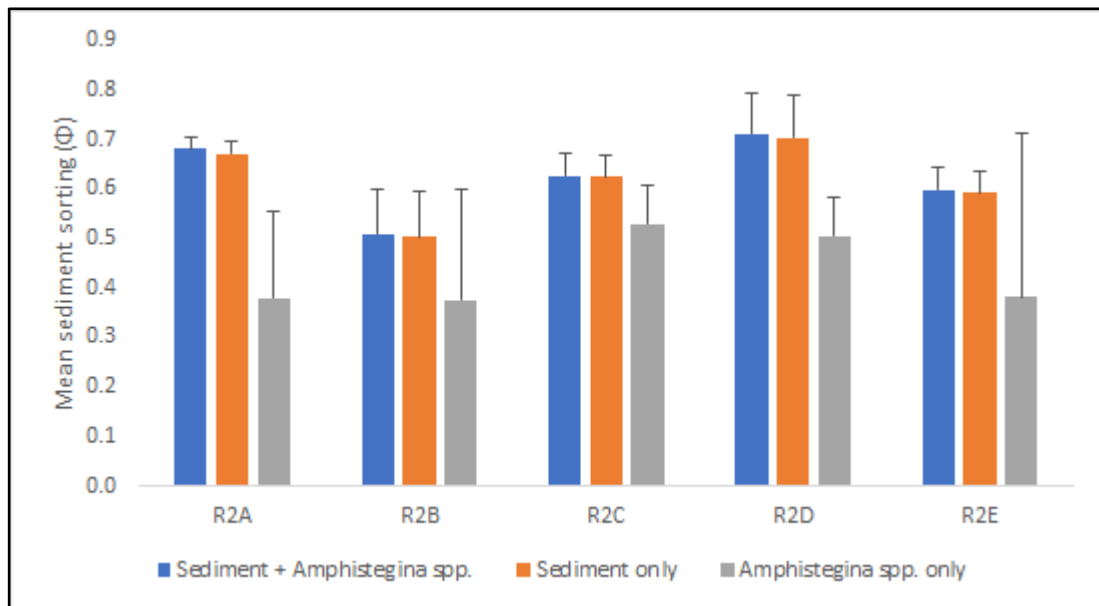
**Figure 3.9.1** – Mean sorting coefficient ( $\Phi$ ) of the different analyzed categories per site. Error bars represent +SD.



**Figure 3.9.2** – Mean sorting coefficient ( $\Phi$ ) of the different analyzed categories in the impacted stations. Error bars represent +SD.

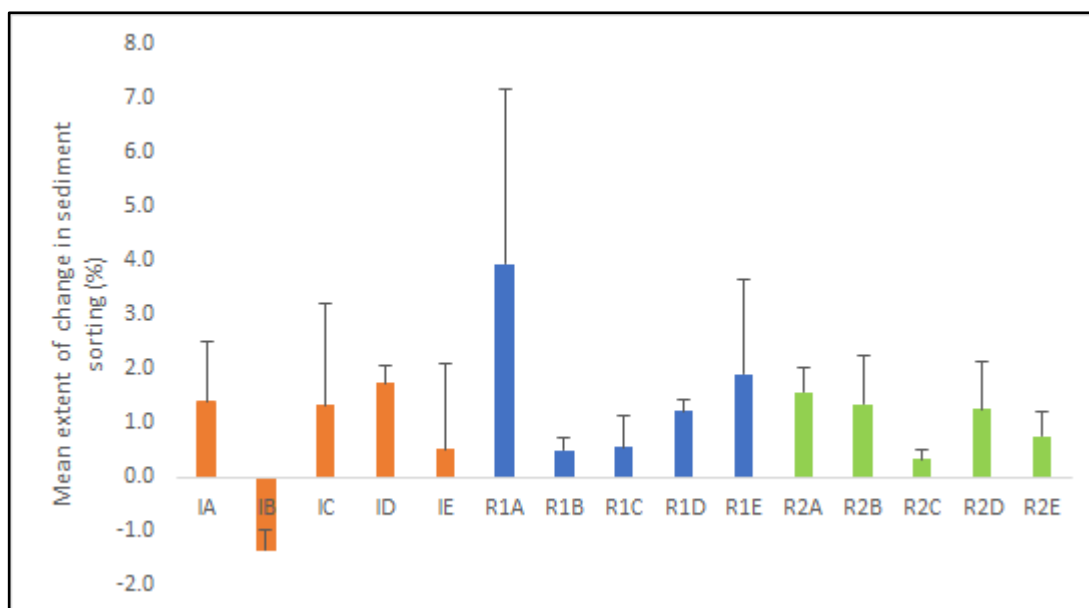


**Figure 3.9.3** – Mean sorting coefficient ( $\Phi$ ) of the different analyzed categories per station in the reference site 1. Error bars represent +SD.



**Figure 3.9.4** – Mean sorting coefficient ( $\Phi$ ) of the different analyzed categories per station in the reference site 2. Error bars represent +SD.

When comparing the extent of change (%) in sediment sorting among the stations (Figure 3.9.5), station IB, which was the one positioned right in front of the outfall (see Figure 2.2.1), had a negative percentage change in sorting value, meaning that in this station the sediment sorting value decreased due to the presence of *Amphistegina* sp.. Although it can be considered as a contributing factor, the absolute abundance of *A. lobifera* does not seem to be the main cause of this difference as the species abundance in station IB was not the highest of all the stations (Figure 3.1.1). Additionally, even though the *A. lobifera* individuals at station IB showed a larger size when compared to individuals from the other stations (Figure 3.2.1), the sediment itself also had a larger mean size (Figure 3.8.2). With that, in IB coarse material was added to sediment already previously coarse, making it better sorted (and hence giving a smaller sorting value). Therefore, taking station IB as an example, when considering the impact of the *A. lobifera* on sediment sorting, it is likely that the ‘initial’ sediment size (i.e. before/without the presence of *A. lobifera*) posed a more significant influence on the extent of change in sediment sorting coefficients in the present study than the size of amphisteginids or their absolute abundance. However, it is still important to highlight that such changes were small (less than ca 2%), and that due to the lack of a statistical significance revealed by the PERMANOVA test (Table 3.9.1), there seems to be no difference when comparing sorting coefficients between the impacted and reference sites in the present study.

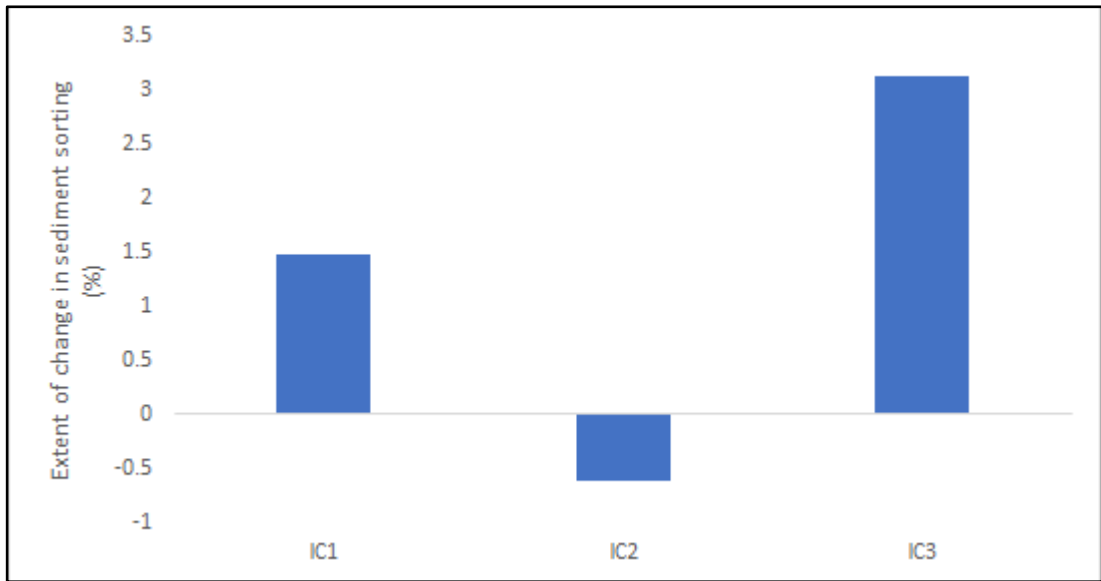


**Figure 3.9.5** – Mean extent of change in sorting (%) due to the presence of *Amphistegina lobifera* per station in the three three analyzed sites. Error bars represent +SD.

**Table 3.9.1** – Permutational multivariate analysis of variance (PERMANOVA) table for differences in extent of change (%) in sorting ( $\Phi$ ) due to the presence of *Amphistegina lobifera* between the impacted and reference sites.

Source of variation	df	Sum of squares (SS)	Mean of squares (MS)	Pseudo-F	P (perm)
Site	2	6.1812	3.0906	0.8107	0.5325
Contrast: impacted site vs reference sites	1	3.7362	3.7362	1.0078	0.3644
Residuals	30	46.663	1.5554	–	–

Besides the lack of statistical significance in sorting differences between the impacted and the reference sites, natural variability in sediment sorting was also observed when comparing the three replicates from each station. At station IC, for example, the replicates IC1, IC2 and IC3 showed small but very different extents of change in sediment sorting with and without *A. lobifera* (Figure 3.9.6). This highlights the possible lack of correlation between high seawater temperature, the spread of amphisteginids and alterations in sediment sorting in the present study.



**Figure 3.9.6** – Extent of change in sediment sorting (%) in the three replicates of station IC, illustrating the natural variability found between the samples' replicates.

## 4. Discussion

### 4.1 Absolute and relative abundances of *Amphistegina lobifera* and relation to sediment granulometric characteristics

Since it was first recorded in the Maltese islands (Yokes *et al.*, 2007), the presence of *A. lobifera* in different areas of the archipelago has been well documented in the literature (Agius, 2018; Guastella *et al.*, 2019). In the present study, the species was recorded for the first time from Il-Ħofra ż-Żgħira, Il-Ħofra I-Kbira and Il-Kalanka it-Tawwalija Bay, which highlights the widespread occurrence of this foraminifer in Malta. However, *A. lobifera* has probably been in the analyzed sites for longer since it was already widespread around the Maltese Islands in 2007 (Yokes *et al.*, 2007). Given that the presence of this species in the Mediterranean is most likely explained by Lessepsian migration (Caruso and Cosentino, 2014b; Guastella *et al.*, 2019), *A. lobifera* probably first reached the East coast of Malta, which includes Il-Ħofra ż-Żgħira, Il-Ħofra I-Kbira and Il-Kalanka it-Tawwalija Bay, and then spread towards the western areas of the Maltese Islands.

The impacted site had a significantly higher absolute abundance of *A. lobifera* compared to the two reference sites (ca 161 ind./g at I, vs 75 ind./g at R1 and 43 ind./g at R2). These differences are not attributable to the measured physicochemical parameters other than temperature (i.e. salinity, conductivity, and pH), since they were all extremely similar in all three bays (see Appendix A for complete results). The bays' geomorphological differences also probably did not affect the results recorded since R2 had a different shape from both I and R1 and still presented a mean absolute abundance of the species similar to that of R1. Nevertheless, the lowest overall absolute abundance in R2 compared to the other two sites might be related to it being furthest from the thermal effluent, while the higher number of amphisteginids at the impacted site may contribute to an increase in abundance in R1 due to the proximity of the two bays – the waters of which are also physically connected through a natural arch, which might facilitate dispersal.

Besides the overall difference in the absolute abundance of *A. lobifera* between the impacted and reference sites, some stations differed from the others at the same site. For example, the lower absolute abundance of *A. lobifera* in station ID, when compared to the other impacted stations, might be related to the mean particle size

at this station, since the mean size of the sediment with and without amphisteginids were smaller in station ID, even though the foraminiferal shells were overall the same size as at the other impacted stations (see Figure 3.8.2). The same explanation might be given to account for the difference in absolute abundance between the three sites, as the sediment with and without amphisteginids had its largest size at I (which was classified as 'coarse sand'), being finer at R1 ('medium sand') and finest at R2 ('medium sand').

A coarse and therefore harder sediment surface is the ideal substratum for epifaunal species (El Kateb *et al.*, 2018), while fine sediment (<63 µm) is not ideal for *Amphistegina* spp. (Murray, 2006 as cited in El Kateb *et al.*, 2018). Thus, amphisteginids have a lower chance of establishing a large population in finer sediment and station ID showed a lower absolute abundance from the other impacted stations, while site I had an overall higher abundance of the species when compared to the less coarse sites R1 and R2. A similar explanation may also be applied to stations R1A and R1E, which showed similar absolute abundances of *A. lobifera* to most of the impacted stations. Even though all R1 stations were classified as 'medium sand', both the mean particle size of sediment with and without amphisteginids were higher in R1A and R1E than in the other R1 stations and, therefore, the establishment of *A. lobifera* in these two stations might have been favoured by the slightly coarser sediment present. The same applies for station R2D. Therefore, in general the species was found more commonly in coarser sediment – i.e. in the impacted site in general, as well as in those reference stations that had slightly coarser sediment than other stations from the same site. This finding is compatible to the situation in other Maltese sites invaded by the species, in which the highest abundances of *A. lobifera* were also recorded in the coarser sediment (Guastella *et al.*, 2019).

Yet, these differences from the overall absolute abundance pattern of each station observed in ID, R1A, R1E and possibly R2D are probably not influenced by the sediment sorting, as the sorting coefficients of sediment with amphisteginids for the four above mentioned stations did not differ significantly from the other stations in their respective sites (see Figures 3.9.2 – 3.9.4). Besides that, it is notable that station IB, the closest one to the thermal effluent outfall, showed the highest mean sorting coefficient from all the analyzed stations for both sediment with and without amphisteginids, meaning that the sediment here was more poorly sorted than in the other stations from all sites. Also, both the sediment with and without amphisteginids was slightly coarser at station IB, which might be related to the current generated by



the thermal effluent, which possibly agitates the surface sediment winnowing away the finer and therefore lighter particles.

Nevertheless, when analyzing the mean extent of change in sorting, the percentage change value was negative for IB (see Figure 3.9.5), meaning that the presence of amphisteginid tests decreased the sediment sorting value. In spite of the closer distance of station IB to the effluent, this difference in sorting does not seem to be driven by the amphisteginid invasion (and, therefore, indirectly, by elevated temperature) due to the lack of statistical significance when analyzing differences in sorting between the three sites. This might also be explained by the overall similarity in sorting coefficients between sediment with and without amphisteginids in all the other stations. In IB, however, the sediment without amphisteginids was already more poorly sorted (higher sorting coefficient) and with a slightly higher mean grain size value than the other stations (see Figure 3.8.2), meaning that the sediment changed from poorly sorted to slightly less poorly sorted, and that coarser particles (coarser amphisteginid shells) were added to an already coarse sediment, making it even coarser.

*Amphistegina lobifera* can be found on both algae and in sediment (Hohenegger, 1994), mostly living on hard and phytal substrates (Triantaphyllou *et al.*, 2012). The sediment sampled in the present study contained individuals that were probably displaced from algae by water motion and that then lived on the bottom sediment. Thus, another factor that probably had an influence on the distribution of *A. lobifera* in the analyzed sites was a possible difference in the occurrence of algal species which the foraminiferans attach to, such as *Sargassum* sp. which is more common in the impacted than in site R1 (Sciberras, 2015), or *Halopteris* sp., which living *Amphistegina* spp. is known to attach to in Maltese waters (Agius, 2018). Possible differences in the distribution of algal cover between the bays might have also influenced the present results, and can be further investigated in future studies.

Furthermore, nutrient flux and water temperature limit the distribution of symbiont-bearing large Foraminifera (Langer & Hottinger, 2000). This is related to the habitat depth, as *A. lobifera* lives predominantly at <20 m depths (Hallock, 1985; Triantaphyllou *et al.*, 2012 and references therein). The differences in depths (see Table 2.2.1) from which samples were collected between stations might have possibly influenced the differences found in absolute and relative abundance of *A. lobifera*. However, it is clear that the depth variation between the analyzed stations was minor

(2.5–6.0 m) and that the mean depths per station were similar (3.58 m for I, 4.40 m for R1 and 4.36 m for R2). The influence of depth on biological aspects of benthic Foraminifera cannot be separated from the influence of other ecological parameters (Boltovskoy *et al.*, 1991). The effect of depth on abundance of *A. lobifera* is related to light intensity, since *A. lobifera* lives mostly in shallow waters (Hallock, 1985; Triantaphyllou *et al.*, 2012 and references therein) and hydrodynamic energy, temperature and light intensity decrease with depth, which is therefore considered as a secondary factor influencing the distribution of large benthic Foraminifera (BouDagher-Fadel, 2008). Additionally, temperature has been shown to exert a larger influence on the distribution of *Amphistegina* spp. when compared to light intensity (Hollaus & Hottinger, 1997). All in all, depth differences do not represent the main source of variation in *A. lobifera* abundances between the studied stations.

In spite of the granulometric composition influence on the spread of amphisteginids in the studied bays, the role played by temperature on the spread of thermophilic species such as *A. lobifera* (Langer & Hottinger, 2000) also has to be taken into consideration. Even though the sediment granulometric characteristics might help to explain the minor differences found between the sites and stations analyzed in the present study, temperature still seems to pose the largest influence on the main differences found in the spread of amphisteginid Foraminifera when comparing a site with higher seawater temperatures than the two reference sites. Additionally, it is also evident that in spite of the sediment being coarser at the impacted site, all three sites were classified in the same granulometric category ('sand'), which reinforces the argument that granulometric differences might help to understand minor differences, but the widespread presence of *A. lobifera* is temperature-driven not only in the analyzed sites but also globally, as discussed in section 1.2.

#### **4.2 The influence of temperature on the spread of *Amphistegina lobifera* in the analyzed sites**

The distribution of *Amphistegina* is limited by the winter sea surface temperature isotherm of 14°C (Zmiri *et al.*, 1974; Larsen, 1976; Langer & Hottinger, 2000), and the central Mediterranean Sea shows a winter isotherm of 15°C (AIS Environmental Ltd., 2011). Thus, survival of amphisteginids in the bay is possible even in colder seasons, and the higher mean absolute abundance of *A. lobifera* in the impacted site when

compared to R1 and R2 matches the expected temperature-related distribution of the genus.

Even though the temperature recordings between the three sites were very similar in the present study (see Appendix A), Il-Ħofra ż-Żgħira (the impacted site) is known to present higher seawater temperatures due to the presence of the thermal effluent from the Delimara Power Station in the bay (see section 1.4). At the impacted site, the minimum reported temperature is of 15°C (Micaela Cassar, *personal communication*, 2018), and the maximum of 30°C (Sciberras, 2015), while for R1 the ranges are of ca 16°C (Micaela Cassar, *personal communication*, 2018) – 25°C (Micaela Cassar, *personal communication*, 2017). For R2, there is no regular monitoring and the data registered in the present study was of ca 22°C for all stations. However, as mentioned in section 1.4, the actual difference in temperature in the impacted site relative to ambient conditions can fluctuate.

Still, such studies are based on either annual averaged data or point samplings, and the lack of regular data from frequent monitoring of the seawater temperature fluctuations can be considered as a limitation to better understanding the temperature patterns in the referred bays. The lack of constant temperature monitoring at Il-Kalanka Bay (R2) might also be a limiting factor to understand the overall differences in temperature throughout the year between the three bays. Even so, the different data recorded at the impacted site, where the thermal effluent is located, reinforces the point that overall temperatures at Il-Ħofra ż-Żgħira are in any case generally higher than in other local areas.

Global ocean temperatures are predicted to rise by 2.6–4.8°C by the end of this century (IPCC, 2013), and the Mediterranean by 2–4°C from 2071–2100 when compared to 1990–2019 (Hertig & Jacobeit, 2007). As discussed in section 1.3, the overall higher temperatures found at the impacted site make it a suitable proxy for gaining an insight on changes likely to be brought about due future climate change-driven marine temperature changes. Considering that the highest abundance of *A. lobifera* was found at the impacted site, one can conclude that the invasion of this thermophilic species in the bay is probably influenced by elevated seawater temperature and, therefore, that climate change is likely to influence the abundance of amphisteginid Foraminifera in Maltese waters, as has been happening not only in

the Mediterranean but also in the global ocean (Weinmann *et al.*, 2013a; Weinmann *et al.*, 2013b).

#### **4.3 The ecological implications of the spread of *Amphistegina lobifera* in the analyzed sites**

The highest relative abundance of *A. lobifera* when compared to other stained Foraminifera in the impacted station corresponds similarly to the absolute abundance results observed. This is likely an indication that the spread of *A. lobifera* in the analyzed sites is displacing the local foraminiferal assemblages, since amphisteginid Foraminifera mass invasions have been recorded to homogenize foraminiferal faunas (Langer *et al.*, 2012; Mouanga & Langer, 2014). Amphisteginid expansions are known to reduce species richness (Mouanga, 2017) and impact native assemblages (Mouanga & Langer, 2014). Their rapid and abundant expansion will most likely cause changes in native ecosystem functioning (Langer & Mouanga, 2016).

For example, due to their size, dead shells of large foraminiferans can also be used by smaller foraminiferans as a stable feeding platform (Martin, 2008). Considering that the sorting values of *Amphistegina* spp. were marginally higher in the two reference sites and, therefore, that the individuals recorded in the impacted site showed more similar-sized tests, one can hypothesize that the availability of such 'feeding platforms' would be different between the impacted and reference sites, which would have different-sized platforms. This could affect the abundance and diversity of smaller foraminiferal species that would be able to use the dead shells for support as their feeding platform.

The values of the extent of change in sorting were, besides not statistically significant, small and apparently did not pose an overall drastic influence on biological changes in the present study. However, the extent of change in mean sediment grain size due to the invasion of *A. lobifera* were significantly different between the impacted and reference sites. Furthermore, *A. lobifera* shells were always bigger than the sediment in all stations. With that one can conclude that, in the long term, *A. lobifera* might alter the sediment composition in the analyzed sites, especially at Il-Hofra ž-Žgħira where the artificially warmer seawater temperature seems to promote larger populations of the invasive foraminiferan.

Finally, in spite of the general negative effect of current ocean warming and acidification on the holobiont health in symbiont-bearing large benthic Foraminifera that has been described in the literature (Doo *et al.*, 2014), including bleaching in *A. lobifera* (Schmidt *et al.*, 2016a), in general the habitat suitability of *Amphistegina* spp. is predicted to increase both for the Mediterranean Sea (Weinmann *et al.*, 2013a) and for the Atlantic and Indo-Pacific Ocean (Weinmann *et al.*, 2013b). This means that *A. lobifera* will most likely continue to thrive in the climate-change driven warmer oceans in the near future. Such a scenario includes the Mediterranean Sea, as the species seems to better tolerate elevated temperatures when compared to ecologically similar foraminiferal species that are native to the Mediterranean (Schmidt *et al.*, 2016b). Yet, even if the reported negative effect of elevated temperature on *A. lobifera* from some studies in fact takes place, *A. lessonii*, which has not yet significantly invaded the Eastern Mediterranean due to the physical barrier created by the low winter temperatures, is actually predicted to be physiologically able to succeed and expand under global warming conditions at the same time as *A. lobifera* reaches its sensitivity threshold for warmer temperatures (Titelboim *et al.*, 2019). In fact, *A. lessonii* has been recorded in the present study, even though in lower abundances than *A. lobifera* and without a clear trend of differences in abundances between the three sites (see Table 3.1.1). This could mean that, regardless of the actual species, *Amphistegina* spp. show a wide expansion capacity in the Mediterranean and in Maltese waters under climate change conditions, and its ecological effects and conservation concern impacts would be of similar magnitude both on a local and a global scale.

It is still important to highlight that when amphisteginids invasions occur in high abundances their ecological impacts probably take place firstly on the micro-ecosystem of which they form part and then, through these changes, the meso-ecosystem might also be affected over time, potentially resulting in significant ecological alterations not yet fully understood to date, in spite of its relatively small size when compared to other epifaunal species that inhabit shallow sea bottoms.

#### **4.4 Greatest spiral diameter (GSD)**

Tests of *A. lobifera* are known to reach 2–3 mm in the Pacific Ocean (Hallock, 1979), and up to 2 mm in the Mediterranean (Triantaphyllou *et al.*, 2012). Therefore, the mean size variation of 1.0–1.5 mm found in the present study matches what has been

described in the literature for mean greatest spiral diameter. As expected, the mean size of amphisteginids obtained through the granulometric analyses correspond to the GSD values found.

Elevated temperatures have been shown to reduce *A. lobifera* shell diameter (Prazeres & Pandolfi, 2016) and growth (% surface area increase) rates (Schmidt *et al.*, 2016a). However, even in highly thermotolerant *A. lobifera* populations, like the one from the Gulf of Aqaba, reduction in growth is expected at around 32°C but not 30°C (Schmidt *et al.*, 2016b). Therefore, in the Maltese populations of *A. lobifera* the thermal threshold has probably not yet been reached and the species' growth has not been hampered by elevated seawater temperatures.

The presence of larger amphisteginid tests in the impacted site when compared to the reference sites can also be explained by the coarser sediment there since, as discussed in the previous section, it becomes easier for amphisteginids to attach and therefore to survive and reach largest sizes. The same conclusion is reached when comparing the GSD and mean size of the sediment with amphisteginids per station, in which coarser stations overall showed larger amphisteginids (see Figures 3.2.1 and 3.8.3).

Another factor to be taken into consideration is water motion, as small juveniles of *Amphistegina* have a lower adhesive power than bigger and adult individuals, and therefore water turbulence can more easily dislodge them from the algae they attach to (Hallock, 1985). Since the effluent outfall generates a variable but relatively medium-strong current when compared to natural currents in the bay, the lack of smaller individuals in the impacted site might be explained as such high energy water flow can only be tolerated by larger individuals.

#### **4.5 Morphological deformities in *Amphistegina lobifera* (irregular keel, lateral asymmetry, T/D ratios and severe abnormalities)**

The presence of an irregular keel and lateral asymmetry are considered as morphological deformities in *A. lobifera* (Yanko *et al.*, 1998), as are abnormalities in the aperture, dorso-ventral asymmetry, segment loss in the peripheral margin, and other varied types of deformities (Sen Gupta, 2007). Such morphological variations comprise a range of deformities that probably pose different impairments to function

of the foraminiferans depending on the area affected and on the degree of the deformity. One can hypothesize that the abnormality might impose physical handicaps that would hamper shell attachment to algae and sediment surfaces, movement, and even the symbionts' activity due to possible light restrictions in thicker areas of the shell. Possibly even reproduction might be affected since *Amphistegina* spp. can reproduce both through sexual production of gametes and asexually through multiple fission (Yokes *et al.*, 2014). In asexual reproduction, the parent's test is discarded after the division of the protoplasm in smaller portions to generate the young individuals (Rottger, 1974), and the release of the juveniles might be impaired by the deformities. The same applies to sexual reproduction, in which the release of gametes (Rottger, 1974) could be hindered.

Despite being widely referred to in the literature, irregular keel and lateral asymmetry, first described by Yanko *et al.* (1998), were two aspects whose assessment can be considered as a pioneering effort in the present study. Yanko's work did not clearly define how the concept of 'irregular keel' was assessed. In the present study, instead, such morphological feature was analyzed based on the division of shells into defined categories (see Figure 2.8.3) in order to more accurately assess keel shape. Even if Yanko *et al.* (1998) actually considered the overall test outline as being irregular or regular without looking at lateral views of tests, it is important to highlight that the classification used in the present study would also be compatible to the concept used in such work – i.e. an irregular keel in the present work's classification would probably also be identified as an irregular keel according to the criteria used in Yanko's work, since the lateral view is also part of the general outline of the shell. Thus, the method used for analyzing keel shape in *A. lobifera* was probably not a source of bias in the presented results.

An increase in temperature seems to be related to intraspecific morphological variations in benthic Foraminifera (Boltovskoy *et al.*, 1991). However, keel shape and lateral asymmetry showed opposite results in the present study when analyzing differences between the impacted and reference sites – only the former being statistically significant. As they are both parameters that involve variations in the general external shape of the shell, they are probably correlated and consequently other environmental factors besides temperature are likely involved in determining the differences found between the analyses of the two deformities. However, it is still important to note that, for keel shape, the counts of irregular keel were significantly higher in I than in R2, and the difference between I and R1 can be considered as

almost significant ( $p = 0.0678$ ), meaning that overall more irregular keels were found at the impacted site. Further studies are required in order to assess the influence of elevated seawater temperature on the extent of irregular keels in *A. lobifera* populations.

Heavy metal pollution has been related to morphological abnormalities in benthic Foraminifera (Alve, 1991; Sharifi *et al.*, 1991; Yanko *et al.*, 1994). When incorporated into the foraminiferal cell, heavy metals interact with cytoskeleton proteins, harming them and consequently causing deformities (Yanko *et al.*, 1998). However, since the entire cell is permeated by cytoskeleton (different parts of it being responsible for the development of different areas of the test), depending on the cytoskeletal area affected by heavy metals, the type of deformities also vary and there is no correlation between the influence of heavy metals and the type of deformity generated (Yanko *et al.*, 1998).

Deformities (especially irregular keel development and lateral asymmetry) in *A. lobifera* have been described to be significantly impacted by a rise of up to 4 ppm in sediment concentration of cadmium (Yanko *et al.*, 1998). Axiak (2013) described cadmium levels at the cooling water discharge point in Il-Ħofra ż-Żgħira of 0.001 ppm, and a more recent monitoring from 2017 shows that the concentration of cadmium in the bay, as an annual average, was of 0.00 – the annual limit being set as of 0.0002 ppm (IPPC Permit for Delimara Power Station, 2017). The concentration of this heavy metal, therefore, does not seem to be a cause of the occurrence of morphological deformities in *A. lobifera* in the impacted population. Nevertheless, it is noteworthy that the mentioned annual averages might not accurately represent the short-term variations in heavy metals discharge in the thermal effluent, which could still represent a possible significant influence on the extent of morphological deformities in the populations in the bay. Besides, more data on the presence of heavy metals, specifically cadmium, for the other sites is needed in order to assess the impact of this type of pollution on these sites.

With regards to lateral asymmetry, different parameters can influence benthic Foraminifera test shape, like temperature, salinity, solubility of calcium carbonate, nutrition, substratum, light, water motion, etc. (Boltovskoy *et al.*, 1991). Nevertheless, there were no significant differences found between the lateral asymmetry ratios in the three sites. This might be due to the overall similarities in the environmental



parameters in the three sites, and due to the fact that temperature does not seem to be described in the literature as being related to changes in lateral asymmetry in *A. lobifera*. Additionally, as discussed above, the low concentration of heavy metals is probably also related to the similar lateral asymmetry ratios found in the three sites.

The similar type of sediment present in the three bays might also be a reason for the similarities in lateral asymmetry ratios. For example, in the case of *Discanomalina semipunctata*, which belongs to the same order as *A. lobifera* (Rotaliida) and has a similar trochospiral coiling (Loeblich and Tappan, 1988), it was shown that for this species the test's lateral asymmetry is related to the type of substratum – i.e. showing perfect bilateral symmetry in sandy or muddy substrates, a plano-convex structure on rocky substrates, and asymmetrical tests on algal (grassy) ones (Medioli & Scott, 1978). Further studies on possible differences in lateral asymmetry ratios when comparing different substrata from the three study inlets can help to further investigate this aspect.

It is still important to mention that, as for irregular keel, the concept used by Yanko *et al.* (1998) to define 'lateral asymmetry' in *A. lobifera* was not clearly described and is apparently related to an overall imbalance in the shell length proportions (being elongated in one half of the greatest diameter instead of being perfectly rounded, for instance; see Figure 1.1.2). However, asymmetry in *A. lobifera* has been differentiated between dorso-ventral asymmetry and lateral asymmetry, which would yet be different from an irregular keel (Sen Gupta, 2007). In the present study, the lateral asymmetry concept used was based on the lateral view of the shell.

Flattening has also been described as a morphological deformity in *A. lobifera* (see review by Boltovskoy *et al.*, 1991). In general, *A. lobifera* tests are very robust, with typical T/D ratios reaching >0.6 (Hallock, 1979). This is similar to the mean T/D ratio of 0.585 found between the three sites in the present study, as well as to the mean T/D ratios of each analyzed station.

Generally, test shapes of *Amphistegina* spp. vary according to depth, being spherical and robust in shallow waters, and thin and flat in deeper areas (Hallock & Hansen, 1979). Laboratory experiments demonstrated that light and water motion can influence *Amphistegina* spp. test shape (Hallock *et al.*, 1986), but that temperature plays the major influence in the depth distribution of the genus (Hollaus & Hottinger,

1997). However, specifically for *A. lobifera*, test shape does not seem to be influenced by depth as the species occur in a narrow depth range (Hallock, 1979). Living in shallower waters, *A. lobifera* is more subject to water motion and therefore requires thicker shells (Kuile & Erez, 1984). In fact, in the present study no significant differences were found between T/D ratios in the analyzed sites, which might be thus explained by the similar sampling depths – which is related to the depth extent of occurrence of the species of 0–130m (Hallock, 1985). Also, once again the other environmental parameters analyzed (i.e. salinity, conductivity, and pH) did not vary significantly between the sites, and temperature did not seem to influence the T/D ratios of the studied populations.

Finally, with regards to the aberrant morphologies observed, overall the type of deformity did not seem to be related to elevated seawater temperature in the present study since different types of deformities were found in all three sites. Notwithstanding, a more detailed analysis on each counted shell would be required in order to assess the quantitative extent of the variation in deformities throughout the whole analyzed populations, since due to time constraints only the severely deformed specimens were picked for abnormality descriptions. Thus, more subtle deformation variants might still be present and further studies are required in order to properly identify and estimate the abundance and extent of the occurrence of such morphological abnormalities in the studied *A. lobifera* populations.

Even though thermal activity is long known to influence benthic Foraminifera (Schafer, 1970; Schafer, 1973), other causes of deformities rather than temperature might also be involved as tests abnormalities in benthic Foraminifera can also be related to natural environmental stress (such as variations in salinity, for example) or even to mechanical damage (Geslin *et al.*, 2000), in which case broken shells show distorted shapes once repaired (Toler & Hallock, 1998). Additionally, morphological deformities in benthic Foraminifera caused by high concentration of metal pollutants have been described not only in cold waters (Alve, 1991; Sharifi *et al.*, 1991) but also in the warmer waters of the Mediterranean (Yanko *et al.*, 1994). Therefore, further studies are required in order to determine the specific causes of the observed aberrant morphologies in the analyzed populations from Malta. For this, the use of scanning electron microscope (SEM) is required to detect abnormalities that are stress-related (Geslin *et al.*, 2000), as well as laboratory experiments to isolate the different factors possibly involved in such abnormal morphological variations. This is also important

as test deformities in benthic Foraminifera can be used as a proxy for marine pollution monitoring and for paleontological studies (Alve, 1995).

#### **4.6 Coiling direction**

Foraminiferal coiling directions might be related to different biological and ecological factors such as life cycle stages or evolutionary and environmental variations (Hallock & Larsen, 1979 and references therein). Different regional populations of *A. lobifera* might present different coiling proportions, and within the same population there is also the possibility of temporal variation in this proportion as the specimens reach maturity (Hallock & Larsen, 1979).

Some Eastern Mediterranean populations of *A. lobifera* are known to be predominantly sinistral (Hallock & Larsen, 1979). Even though in the present study the proportion of sinistral/dextral individuals between the three sites was similar (not statistically significant), the fact that the great majority (at least ca 90%) of specimens found were sinistral in all stations is comparable to the above mentioned results from elsewhere in the Mediterranean.

A higher incidence of sinistral tests of *A. lobifera* has been reported under a 4°C above ambient thermal effluent (Muller, 1977). Age-dependent coiling was also observed by Hallock & Larsen (1979), who found more sinistral *A. lobifera* in adult size classes, similarly to what has been found for the Mediterranean population in the present study. Hallock & Larsen (1979) observed different mortality rates depending on the coiling direction of the individuals (the 'majority coiling' direction group seemed to reproduce earlier than the minority one), leading to an age-specific differential mortality between sinistral and dextral *A. lobifera*. Even though *A. lobifera* age was not assessed in the present study, the largest size group can be assumed as the mature one (as done in Hallock & Larsen, 1979), and this might have been a source of variation in the obtained coiling results.

However, no differences were found in coiling directions between the three sites and, therefore, temperature did not appear to pose a significant influence in the coiling direction of *A. lobifera* in the present study.

#### 4.7 General conclusions

- Both relative and absolute abundances of *A. lobifera* were higher in the impacted site when compared to the reference sites. The colonization of *A. lobifera* in the studied sites seems to be temperature-driven and there are indications that the alien may be displacing local foraminiferal species;
- There were no significant differences found in thickness/diameter ratios, lateral asymmetry and coiling direction between the impacted and reference sites. Overall most of the specimens in the present study were sinistrally coiled;
- Different types of severely aberrant morphologies did not seem to be related to elevated seawater temperature;
- Tests were significantly larger and with a higher incidence of irregular keels in the impacted than the reference sites, which might be related to the higher seawater temperature in the former;
- The invasion of *A. lobifera* is changing the local sediment granulometric characteristics at the impacted site and, in the long-term, this may lead to significant alterations both in physico-chemical and ecological characteristics of the analyzed areas;
- The findings of the present study match well with what has been described for the Mediterranean Sea with regards to climate change-driven amphisteginid invasions. Due to its elevated seawater temperature, the studied impacted site, Il-Hofra ż-Żgħira, can be used as a proxy to better understand climate change impacts on marine benthic foraminiferal species.

#### 4.8 Future work

As an extension of the present study, future research could be carried comparing the recorded absolute and relative abundances of amphisteginids in the studied sites with samples collected during summer in order to assess whether there are significant seasonal variations in the analyzed amphisteginid populations. Studies of different amphisteginid habitats at the three sites and their possible influence on the foraminiferal abundance could also be carried out; comparing, for example, sediment and rocky habitats, or assessing differences in amphisteginid abundance in relation to the extent of algal coverage.

Assessment of morphological variation and the relationship with temperature through the use of scanning electron microscope (SEM) might also help to detect stress-related abnormalities (Geslin *et al.*, 2000). It is also possible to isolate, via laboratory experiments, some of the possible environmental factors influencing the analyzed biological aspects of amphisteginids (e.g. artificially maintaining living specimens under different conditions of temperature, pH, salinity, etc.), in order to determine possible impacts on their reproduction, growth, bleaching, morphological features etc. These biological parameters directly influence the foraminiferans' ability to cope with and survive under elevated temperature conditions and can, therefore, be possibly used to estimate their ability to thrive under future climate change conditions.

Research on the analyzed foraminiferal assemblages that takes place over a longer period of time is also desirable in order to better understand the local scenario involving the biological parameters considered in the present study. The same applies for community studies, which could also assess other species of Foraminifera with regards to the impacts of temperature and other uncontrolled variables on these organisms.

Further studies on the ecological impacts of very small alien species, like the impacts of amphisteginids on benthic foraminiferal microhabitats, are required in order to better characterise the extent and impact of invasions by very small organisms such as foraminiferans. In spite of their sometimes microscopic size, *A. lobifera* seems to cause profound ecological alterations in the micro and meso-ecosystems they occur in – especially under accelerated climate change conditions.

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