

Chromatography  
and  
DNA  
analysis  
in  
archaeology

Editors

CÉSAR OLIVEIRA  
RUI MORAIS  
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# ArchaeoAnalytics

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## ANCIENT DNA IN ARCHAEOLOGICAL GARUM REMAINS FROM THE SOUTH OF PORTUGAL

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### ABSTRACT

The recent discovery of *garum*<sup>1</sup> remains in a Roman archaeological site in the south of Portugal, used for the processing and storage of fish and shellfish-based products, offers an unprecedented opportunity to pioneer biological DNA-based analyses of archaeological remains, through cutting-edge analytical methods like *High Throughput Sequencing* (HTS). Our study seeks to reconstruct the species composition of the seafood products used in *garum* preparation of this region over the centuries IV and V, applying a combination of the DNA barcode approach with HTS technology. Initially, primer combinations amplifying different and short fragments (<320 bp) of the cytochrome oxidase I mitochondrial gene (COI) were tested for success in amplification of DNA

isolated from fish tissue. The most successful primer pair was then used to generate amplicons from 2 *garum* DNA isolates, and submitted to HTS sequencing in a GS 454 FLX Titanium platform. Probably due to the old age of the DNA under examination, only a small number of reads from each sample (<200) was eligible for analyses. These reads were compared to our local comprehensive reference libraries of annotated DNA barcodes for fish and shellfish. We found matches with between 99–100% similarity in seven different fish species. The fish species found are common in the region according to current knowledge. Replicate and additional analyses are being carried out in order to verify and deepen these findings.

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## INTRODUCTION

The study of Roman civilization has captivated the attention and fascinated numerous academics since ancient times. Classically based on historical written resources and iconographies, and on the analysis of archaeological remains, the study of ancient cultures is now facing a new Era. Research fields like paleogenetics approach investigation of the past by encrypting the content of archaeological fragments through analytical techniques, integrating historical, archaeological and empirical approaches in an innovative way (Hansson and Foley, 2008, 1169; Foley *et al.*, 2012, 389; Oliveira *et al.*, 2013, 263).

The availability of ancient objects constitutes therefore an invaluable source of information to understand history, culture, and commerce habits of former societies, information otherwise relegate to the past. Ceramic containers were widely used by ancient civilizations to store and/or transport food items such as wine, honey, olive oil (Hansson and Foley, 2008, 1169) or fish and its derivatives, as *garum* (Foley *et al.*, 2012, 389; Oliveira *et al.*, 2013, 263). These containers made out of clay have a porous consistency that renders them a reservoir of biological material, absorbed and clutched for Centuries (Hansson and Foley, 2008, 1169). By holding organic substances and preserving traces of organic materials from their original content, still nowadays these remains store a unique memory, providing exceptional biological evidences on what the ancient content was. Pioneer chemical analyses on the content of four types of Lusitanian amphorae collected in Portugal and Galicia (Spain), based on the use of Gas Chromatography coupled with Mass Spectrometry (GC/MS), detected residues of fish-based products. These results witnessed that fish was a relevant part of the Roman culture, as reported in ancient texts (Morais *et al.*, in press). Highly fat content fish species, like anchovies, mackerel, sardines, tuna and sprats, were used to prepare *garum*, being fermented and probably mixed with other marine fauna, salt, and plants (Curtis, 2009, 712S; Oliveira *et al.*, 2013, 263).

Information from analytical chemical studies (Oliveira *et al.*, 2013, 263) and archaeological observations (Morais *et al.*, in press) can be complemented and detailed through the analyses of vestiges of ancient DNA from fish or fish derivatives. Though relatively recent, the study of ancient DNA plays a relevant role in exploring spatio-temporal changes of marine fish (Riccioni *et al.*, 2010, 2102), and in analysing remains of ancient organisms by means of genetic tools (Hansson and Foley, 2008, 1169; Rizzi *et al.*, 2012, 1). The hurdles associated to the analyses of ancient, degraded, DNA from unknown samples through classical methodologies has been recently bypassed, for the synergistic power of new high-throughput parallel sequencing, the availability of markers for the analysis of short DNA sequences (mini-barcodes), and of public, globally accessible genetic databases (*e.g.* Barcode of Life Data System; Ratnasingham and Hebert, 2007, 355). High throughput sequencing (HTS) platforms have revolutionized genetic analyses of ecological communities (metabarcoding) or environmental DNA samples (environmental barcoding), allowing taxon identification down to species level in complex mixtures of DNA from multiple organism sources (Shokralla *et al.*, 2012, 1794; Taberlet *et al.*, 2012, 1789). These approaches find robust support in the availability of detailed reference libraries of validated DNA barcodes. Covering taxonomically diverse organisms, reference DNA barcode libraries (*e.g.* Costa *et al.*, 2012, e35858) allow verification of matches between mini-barcodes to full length DNA barcodes identifying a wide spectrum of taxa, and enabling taxonomic resolution of the sequences.

Our study seeks to take advantage of the known potential of combined HTS and DNA barcoding approaches to determine the seafood species composition present in *garum* or other ancient products consisting of organism mixtures. An annotated and validated reference library of DNA barcodes of European fish and marine invertebrate species (*e.g.* Costa *et al.*,

2012, e35858; Kneblsberger *et al.*, 2014, 1060; Landi *et al.*, 2014, e106135; Matzen da Silva *et al.*, 2011, e1944) will be used as a backbone for species identification. Here we report the first trials of our study, consisting on the attempt to identify seafood species present in *garum* by means of amplification of short fragments of the mitochondrial gene cytochrome oxidase I (COI) from *garum*'s DNA extracts, HTS sequencing, and sequence identity match against the reference library. The full-length DNA barcode is too long (about 652 bp) for amplification from ancient DNA (Shokralla *et al.*, 2011, e21252; Taberlet *et al.*, 2012, 1789), therefore primers for amplification of shorter barcode segments (mini-barcodes) were used.

## MATERIAL AND METHODS

### *Sample collection*

A partially buried *dolium* was found in *Boca do Rio*, Budens, Algarve, south of Portugal, in a context of the late Roman era to the end of the 4th century or early 5th century (Figures 1.a and 1.b). The top of the *dolium* (Figure 1.b) was covered with soil and the lower half packed with organic material remains. Scales, teeth and fish bones were identified within this material. Samples were collected in aluminum foil and transported to the laboratory at room temperature.

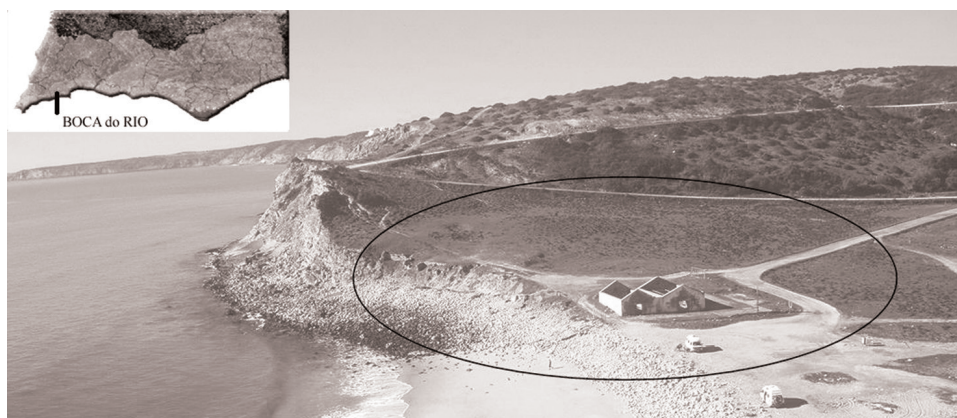


Figure 1.a - Boca do Rio, Budens, Algarve, south of Portugal.

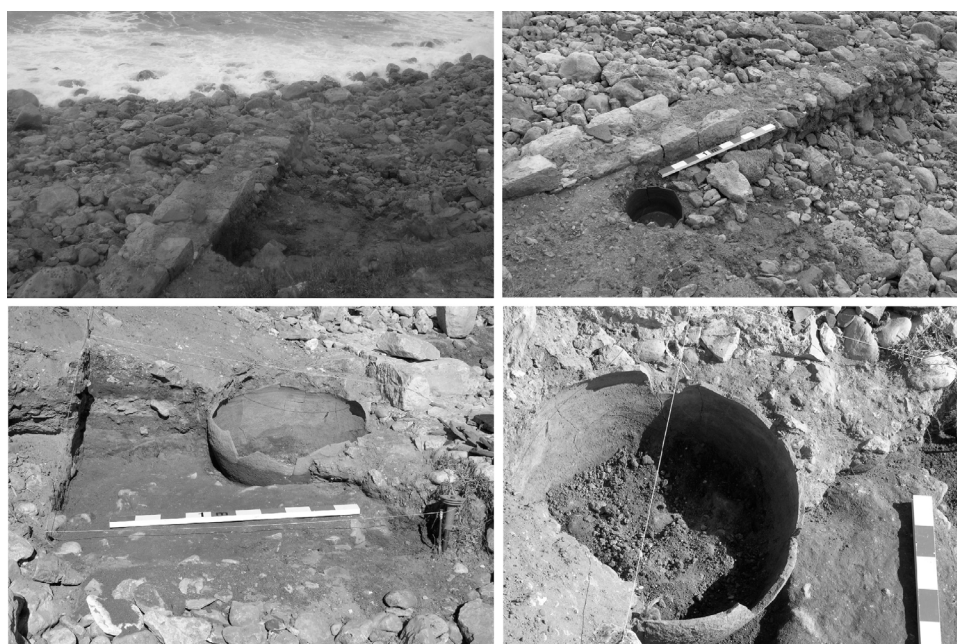


Figure 1.b - Dolium where the content studied was recovered from.

### DNA extraction

Present-day fish and mollusk tissues samples were used as positive controls for testing primer amplification success (detailed below). Tissue from three different fish species and one mollusk species were used – *Auxis rochei rochei* (Risso, 1810), *Sarda sarda* (Bloch, 1793), *Scomber scombrus* (Linnaeus, 1758) and *Calliostoma zizyphinum* (Linnaeus, 1758), respectively. PluralDNA extractions were performed with conventional laboratory methods using *E.Z.N.A Molluscs* DNA Kit (Omega Bio-Tek) and following the manufacturer's protocol.

DNA extractions from *garum* samples were performed under sterile conditions following the manufacturer's instructions for the High Pure PCR Product Purification (Roche) kit. In order to avoid possible contaminations, extractions were carried out in a separate laboratory where marine organisms are not manipulated, located in a different building complex and using exclusive tools and reagents. DNA was isolated from five sub-samples ( $G_1$  to  $G_4$  and  $G_8$ ) of the *garum* material.  $G_1$  and  $G_2$  were composed by undifferentiated material from the whole sample.  $G_3$ ,  $G_4$  and  $G_8$  were composed by scales, vertebrae and teeth fragments, respectively.

### Preliminary tests and primer selection

Different combinations of mini-barcodes primer pairs (Table 1) were tested with DNA extracts from the positive controls listed above in order check their ability to successfully amplify DNA from present-day tissue. The mini-barcode primer combination mlCOIintF – LoboR1 (Leray *et al.* 2013, 34; Lobo *et al.* 2013, respectively) provided DNA amplifications for all tested samples (Figure 2). This primer combination amplifies a 313 bp fragment located in the 5' end of the COI barcode region. Polymerase chain reaction (PCR) thermal cycling conditions were: i) 94°C (5 min); ii) 6 cycles: 94°C (30 s), 62°C (30 s), 72°C (1 min); iii) 35 cycles: 94°C (30 s), 46°C (30 s), 72°C (1 min); iv) 72°C (10 min). Each reaction contained 2.1 µl PCR Buffer (10X), 1.25 µl dNTP mixture (0.2 mM), 0.85 µl of primer mlCOIintF (10 µM), 1.25 µl of primer LoboR1 (10 µM), 0.2 µl DreamTaq DNA Polymerase (5 U/ µl) (Thermo Scientific), 4 µl DNA template, and completed with sterile milliQ-grade water to 25 µl final volume.

Primers	Primer Sequence (5'-3')	Expected product size (bp)	Reference
<b>LoboF1</b>	KBTCHACAAAYCAYAARGAYATHGG	319	Lobo <i>et al.</i> , 2013, 34
<b>mlCOIintR</b>	GGRGGRTASACSGTTCASCCSGTSCC		Leray <i>et al.</i> , 2013, 34
<b>lgLCO1490</b>	TITCIACIAAYCAYAARGAYATTGG	319	Geller <i>et al.</i> , 2013, 851
<b>mlCOIintR</b>	GGRGGRTASACSGTTCASCCSGTSCC		Leray <i>et al.</i> , 2013, 34
<b>mlCOIintF</b>	GGWACWGGWTGAACWGTWTAYCCYCC	313	Leray <i>et al.</i> , 2013, 34
<b>LoboR1</b>	TAAACYTCWGGRTGWCCRAARAAYCA		Lobo <i>et al.</i> , 2013, 34
<b>mlCOIintF</b>	GGWACWGGWTGAACWGTWTAYCCYCC	313	Leray <i>et al.</i> , 2013, 34
<b>lgHCO2198</b>	TAIACYTCIGGRTGICCRAARAAYCA		Geller <i>et al.</i> , 2013, 851

Table 1 - Primer combinations used in this study. For each combination the name, sequence, expected product size, and reference is reported.



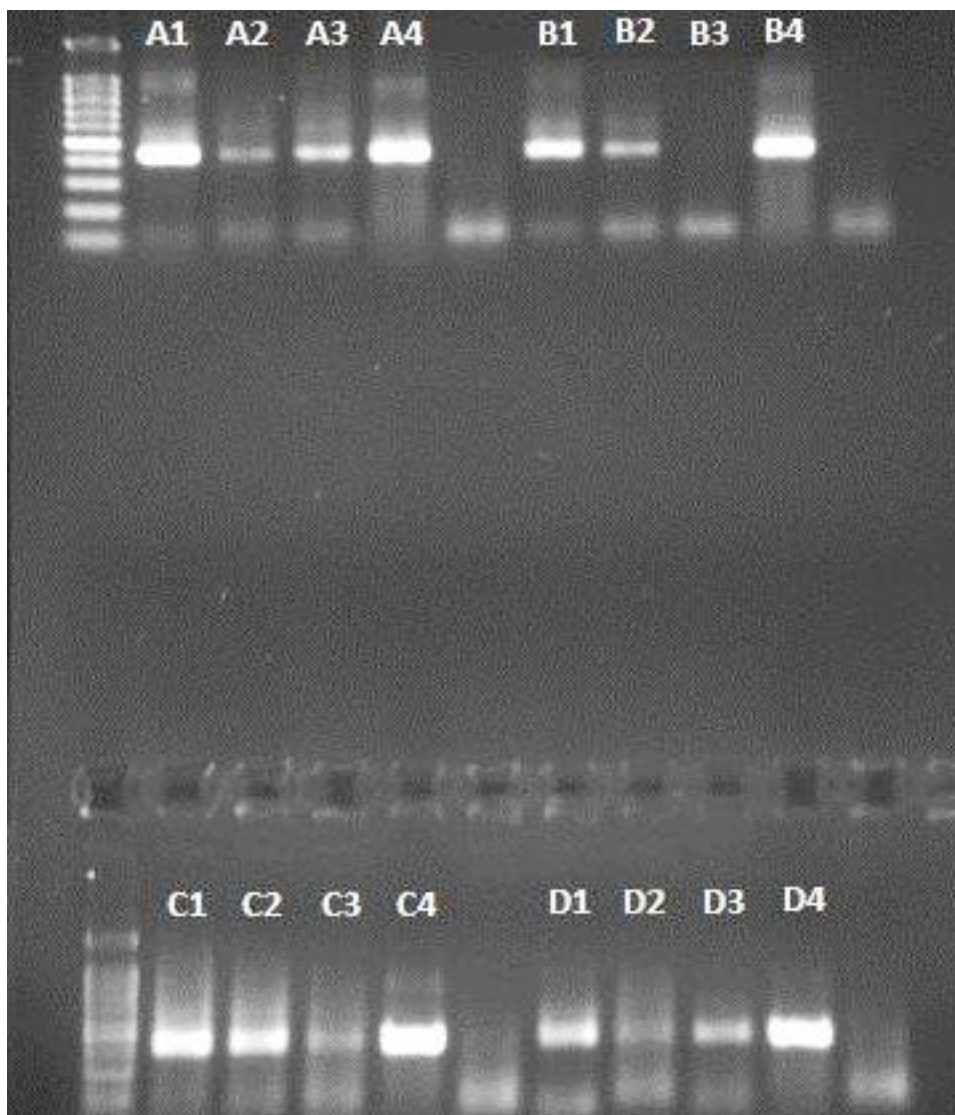


Figure 2 - Agarose gel (1.5%) electrophoresis of modern DNA where A) Primer combination mlCOLintR-LoboF1; B) Primer combination mlCOLintR-jgLCO1490; C) Primer combination mlCOLintF-LoboR1; D) Primer combination mlCOLintF-jgHCO2198; and 1) *Auxis rochei rochei*; 2) *Sarda sarda*; 3) *Scomber scombrus*; 4) *Callistoma zizyphinum*. Lane 1: DNA Ladder (1kb).

### **Ancient DNA amplification**

*Garum* DNA extracts ( $G_1$ ,  $G_2$ ,  $G_3$ ,  $G_4$  and  $G_8$ ) were prepared for HTS sequencing at GenoInseq (Cantanhede, Portugal). PCR amplification of the 313 bp COI fragment was made with fusion primers containing the Roche-454 A and B Titanium sequencing adapters, an eight-base barcode sequence in the fusion primer A, the forward primer mlCOLintF and the reverse primer LoboR1. COI was amplified by PCR in 40  $\mu$ L reactions with Advantage Taq (Clontech) using 0.2  $\mu$ M of each primer, 0.2 mM dNTPs, 1X polymerase mix and 6% DMSO.

The PCR conditions were: i) 94°C (5 min); ii) 6 cycles: 94°C (30 s), 62°C (-1 per cycle) (30 s) and 68°C (60 s); iii) 40 cycles: 94°C (30 s), 46°C (30 s) and 68°C (60 s); iv) 68°C (10 min).

### **Data processing**

The amplicons of  $G_1$  and  $G_3$  were quantified by fluorimetry with PicoGreen (Invitrogen, CA, USA), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to manufacturer's instructions (Roche, 454 life sciences, Branford, CT, USA) at Biocant (Cantanhede, Portugal).

The raw pyrosequencing reads were processed using an automatic pipeline implemented at GenoInseq. In a first step, sequencing reads were assigned to the appropriated samples based on the respective barcode. Then, reads were quality filtered to minimize the effects of random sequencing errors, by the elimination of sequence reads with <150 bp and sequences that contained more than two undetermined nucleotides. Sequences were additionally cut for the reverse primer if present. The results from the filtering step are presented in Table 2.

Each read was individually submitted to the BOLD database identification system (BOLD-IDS), where our reference barcodes are archived and, in parallel, submitted to GenBank's BLASTn (Altschul *et al.*, 1990, 403), in order to search for matching sequences and attempt taxonomic identification.

Sample	Sequenced reads	Sequences after quality filtering	Filtered reads (%)	Discarded reads (%)
G1	1.254	182	14.51%	85.49%
G3	1.780	151	8.48%	91.52%

Table 2 - Filtering step of data analysis.

## RESULTS AND DISCUSSION

These preliminary results were based on a single HTS run, using only one primer pair and one DNA extraction methodology. Therefore, current results await validation and deeper analyses to get a full picture of the *garum* composition. It is well established that the capacity to detect organisms in an environmental sample may be affected by the DNA extraction protocols used, or biased by the relative taxa selectivity of the primer pairs used in PCR (Shokralla *et al.*, 2012, 1794; Taberlet *et al.*, 2012, 1789). Therefore we intend to replicate our current analyses, using additional sub-samples of *garum*, testing different procedures for DNA extraction and applying several primer combinations.

All of the *garum* DNA extracts were amplified for COI with the forward primer mlCOIintF and the reverse primer LoboR1. The agarose gel showed that all samples produced smears indicative of unspecific amplification, a quality criteria necessary to proceed for sequencing. However, G<sub>1</sub> and G<sub>3</sub> were admitted for HTS sequencing due to the nature of DNA and the presence of a fragment with the expected size (Figure 3). After the reads' filtering procedure, both cases resulted in a very low number of eligible reads (Table 2), probably due to the old age of the DNA under study. The sample G1 presented only 14.51% of reads eligible for analysis, while only 8.48% of the reads were accepted for G3. Nevertheless, both HTS runs resulted together in 323 eligible sequences after quality filtering (Table 2). Similarity searches in BOLD (reference library) and Genbank resulted in high similarity matches for 236 of these reads. Ninety-one reads enabled the identification of 7 fish species with 99-100% of similarity. All of them are contemporaneous fish species known to occur and to be common on the Portuguese coast, comprising six species of bony fish and one large sized shark.

In fact, the exploitation and processing of seafood resources in Roman times in the South coast of Portugal is well witnessed (Figure 4). As the archaeological site of *Boca do Rio*, which has registered an indentation of the shoreline well documented since the late 19<sup>th</sup> century (Figure 5), several of these sites are nowadays threatened by the advancing sea. Some of these sites have allowed to detect exploitation of an abundant malacological fauna, as well as bony fish species that these results seem to confirm.

In order to validate and deepen these preliminary results, additional analyses are being carried out, replicating the current analyses, using additional sub-samples of *garum*, testing different procedures for DNA extraction and trying novel primer combinations.

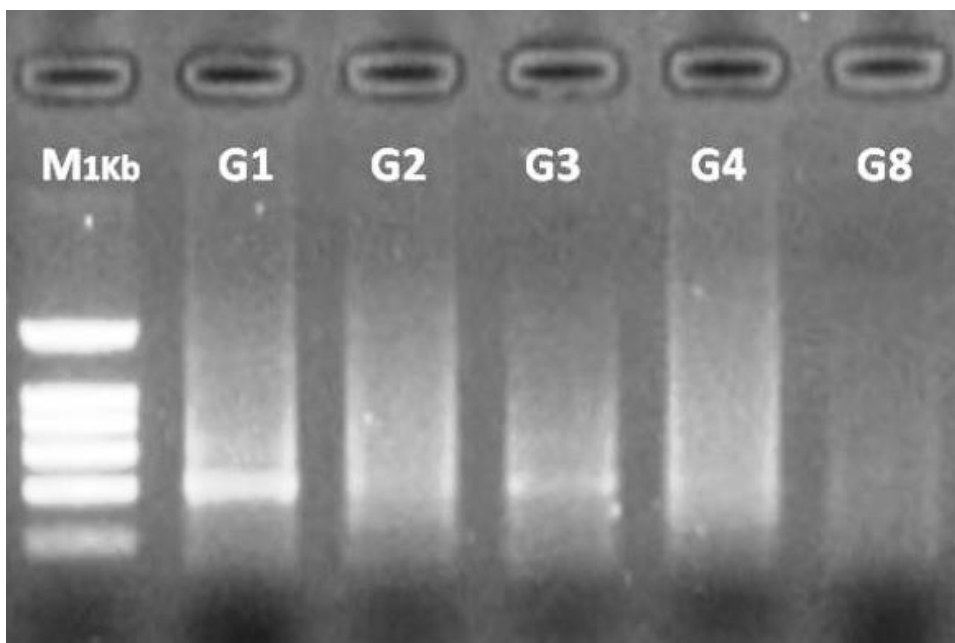


Figure 3 - Agarose gel (1.5%) electrophoresis of the ancient DNA amplified with COI primers. Slight bands observed in G1 and G3 suggests the presence of ancient DNA.



Figure 4 - The Portuguese south Atlantic coast and the location of archaeological roman sites linked to the exploitation of fish preparations.

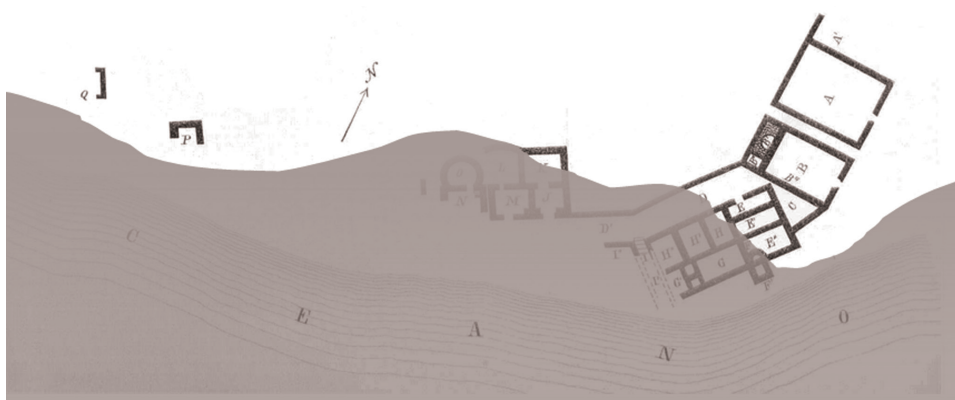


Figure 5 - The archaeological site of Boca do Rio showing an indentation of the shoreline since the late 19th century.

## NOTES

*Garum* is a generic name whose composition varied over time. We apply here the concept as a synonym of fish preparations.

## BIBLIOGRAPHY

- ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W., LIPMAN, D.J. (1990): "Basic local alignment search tool", *Journal of Molecular Biology*, 215, pp. 403–410.
- COSTA, F.O., LANDI, M., MARTINS, R., COSTA, M.H., COSTA, M.E., CARNEIRO, M., ALVES, M.J., STEINKE, D., CARVALHO, G.R. (2012): "A DNA barcode reference library for marine fish of Portugal: design and potential applications", *PLoS ONE*, 7, pp. e35858.
- CURTIS, R.I. (2009): "Umami and the foods of classical antiquity", *American Journal of Clinical Nutrition*, 90, pp. 712S–718S.
- FOLEY, B.P., HANSSON, M.C., KOURKOUMELIS, D.P., THEODOULOU, T.A. (2012): "Aspects of ancient Greek trade re-evaluated with amphora DNA evidence", *Journal of Archaeological Science*, 39, pp. 389–398.
- GELLER, J., MEYER, C., PARKER, M., HAWK, H. (2013): "Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys", *Molecular Ecology Resources*, 13, pp. 851–861.
- HANSEN, A.J., MITCHEL, D.L., WIUF, C., PANIKER, L., BRAND, T.B., BINLADEN, J., GILICHINSKY, D.A., RØNN, R., WILLERSLEV, E. (2006): "Crosslinks rather than strand breaks determine access to ancient DNA sequences from frozen sediments", *Genetics*, 173, pp. 1175–1179.
- HANSSON, M.C., FOLEY, B.P. (2008): "Ancient DNA fragments inside Classical Greek amphorae reveal cargo of 2400-year-old shipwreck", *Journal of Archaeological Science*, 35, pp. 1169–1176.
- IVANOVA, N.V., ZEMLAK, T.S., HANNER, R.H., HEBERT, P.D.N. (2007): "Universal primer cocktails for fish DNA barcoding", *Molecular Ecology Notes*, 7, pp. 544–548.
- KNEBELSBERGER, T., LANDI, M., NEUMANN, H., KLOPPMANN, M., SELL, A.F., CAMPBELL, P., LAAKMANN, S., RAUPACH, M.J., CARVALHO, G.R., COSTA, F.O. (2014): "A reliable DNA barcode reference library for the identification of the North European shelf fish fauna", *Molecular Ecology Resources*, 14, pp. 1060–1071.
- LANDI, M., DIMECH, M., ARCULEO, M., BIONDO, G., MARTINS, R., CARNEIRO, M., CARVALHO, G.R., LO BRUTTO, S., COSTA, F.O. (2014): "DNA Barcoding for Species Assignment: The Case of Mediterranean Marine Fishes." *PLoS ONE*, 9, pp. e106135.
- LERAY, M., YANG, J.Y., MEYER, C.P., MILLS, S.C., AGUDELO, N., RANWEZ, V., BOEHM, J.T., MACHIDA, R.J. (2013): "A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents" *Frontiers in Zoology*, 10(1), pp. 34.
- LOBO, J., COSTA, P.M., TEIXEIRA, M.A., FERREIRA, M.S., COSTA, M.H., COSTA, F.O. (2013): "Enhanced primers for amplification of DNA barcodes from a broad range of marine metazoans" *BMC Ecology*, 13(1), pp. 34.
- MATZEN DA SILVA, J., CREER, S., DOS SANTOS, A., COSTA, A.C., CUNHA, M.R., COSTA, F.O., CARVALHO, G.R. (2011): "Systematic and evolutionary insights derived from mtDNA COI barcode diversity in the decapoda (crustacea: malacostraca)" *PLoS ONE*, 6, pp. e1944.
- MORAIS, R., OLIVEIRA, C., ARAÚJO, A. "The Lusitanian amphorae in Augustan Era", in "International Congress Lusitanian Amphorae – Production and Difusion", Tróia, Portugal, *British Archaeological Reports*, in press.
- OLIVEIRA, C., KUZNIARSKA-BIERNACKA, I., NEVES, I.C., FONSECA, A.M., PARPOT, P. (2013): "Chemical analysis of organic residues in amphorae from the Baetica shipwreck of Esposende". In: Morais R, Granja H, Cerdan AM Eds. *O irado Mar Atlântico. O naufrágio Bético Augustano de Esposende (Norte de Portugal)*, pp. 263–281.
- RATNASINGHAM, S., HEBERT, P.D.N. (2007): "BOLD: The Barcode of Life Data System ([www.barcodinglife.org](http://www.barcodinglife.org))", *Molecular Ecology Notes*, 7, pp. 355–364.
- RICCIONI, G. & LANDI, M., FERRARA, G., MILANO, I., CARIANI, A., ZANE, L., SELLA, M., BARBUJANI, G., TINTI, F. (2010): "Spatio-temporal population structuring and genetic diversity retention in depleted Atlantic bluefin tuna of the Mediterranean", *PNAS USA*, 107, pp. 2102–2107.

- RIZZI, E., LARI, M., GIGLI, E., DE BELLIS, G., CARAMELLI, D. (2012): "Ancient DNA studies: new perspectives on old samples", *Genetics Selection and Evolution*, 44, pp. 1-19.
- SHOKRALLA, S., ZHOU, X., JANZEN, D.H., HALLWACHS, W., LANDRY, J-F., JACOBUS, L.M., HAJIBABEI M. (2011): "Pyrosequencing for Mini-Barcoding of Fresh and Old Museum Specimens", *PLoS ONE*, 6(7), pp. e21252.
- SHOKRALLA, S., SPALL J., GIBSON, J.F., HAJIBABEI, M. (2012): "Next-generation DNA sequencing technologies for environmental DNA research", *Molecular Ecology*, 21, pp. 1794-1805.
- TABERLET, P., COISSAC, E., HAJIBABEI, M., RIESEBERG, L.H. (2012): "Environmental DNA", *Molecular Ecology*, 21(8), pp. 1789-1793.