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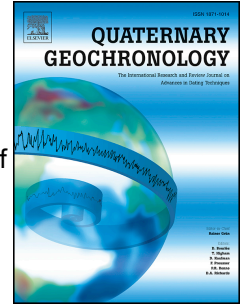
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Pecten as a new substrate for ¹⁴C dating: the Quaternary raised beaches in the Gulf of Corinth, Greece

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1 *Pecten* as a new substrate for IcPD dating: the Quaternary raised 2 beaches in the Gulf of Corinth, Greece.

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13 **Abstract**

14 Intra-crystalline protein diagenesis (IcPD), a recent development of amino acid racemization
15 dating (AAR), is now established as a reliable geochronological tool for the Quaternary.
16 However, extending the method to new biominerals requires extensive testing in order to
17 provide evidence for the closed-system behaviour of the intra-crystalline proteins and to
18 assess the temporal span that can be covered.

19 Here we present results from high-temperature experiments on the IcPD of the bivalve
20 *Pecten*, demonstrating that a fraction of proteins can be isolated from a bleach-resistant
21 mineral matrix, which effectively operates as a closed system under conditions of accelerated
22 diagenesis in the laboratory. Analyses of *Pecten* from the well-dated terrace system of the
23 Gulf of Corinth (Greece) provided a pilot test for the integrity of the intra-crystalline fraction
24 in subfossil shells. The small sample sizes in this preliminary study preclude a full
25 assessment of the aminostratigraphic power of *Pecten* IcPD, but a concordance is observed
26 between the extent of IcPD and sites dating from between MIS 5 and MIS 11.

27 We conclude that *Pecten* is a potentially good substrate for IcPD dating in the Mediterranean,
28 and that the temporal limit of the technique in this area lies beyond MIS 11.

29 **Highlights**

- 30 • A 48-h bleaching step isolates the intra-crystalline fraction of proteins from the
31 modern marine bivalve *Pecten*.
- 32 • The intra-crystalline proteins behave as an effectively closed system during artificial
33 diagenesis.
- 34 • The patterns of diagenesis are predictable, but may differ between high temperature
35 experiments and normal burial temperatures.
- 36 • The extent of IcPD was determined for *Pecten* within a restricted geographical area
37 (the Gulf of Corinth, Greece).
- 38 • There is a concordance between the extent of IcPD and correlated age estimates back
39 to MIS 11 in this region, although the resolution decreases beyond MIS 7.

40 **Keywords**

41 Intra-crystalline proteins; *Pecten* (scallop) shell; amino acid racemization (AAR); dating;
42 raised beaches; Mediterranean.

43 **1. Introduction**

44 *1.1 A new substrate for IcPD dating*

45 Several studies have used amino acid racemization (AAR) geochronology to obtain age
46 information for Quaternary raised beaches in the Mediterranean basin (Hearty, 1986, 1987;
47 Hearty et al., 1986; Belluomini and Delitala, 1988; Hearty and Dal Pra, 1992; Torres et al.,
48 2000, 2010). We have recently reported on the suitability of the bivalve *Glycymeris* for intra-
49 crystalline protein diagenesis (IcPD) dating, concluding that while bleaching isolates an intra-
50 crystalline protein fraction, this fraction may not operate as a closed system in fossil samples
51 of this taxon, identified by comparing the diagenetic patterns of free and total hydrolysable
52 (FAA vs THAA) amino acids (Demarchi et al., 2015). As part of the same study on IcPD
53 dating of Mediterranean shorelines (“mAARiTIME”), we tested a new substrate, the bivalve
54 *Pecten*, which had been successfully used by Murray-Wallace and colleagues (1996; 2005) to
55 build AAR chronological frameworks using whole shell (i.e. unbleached) back to MIS 8 for
56 low shelf deposits in Australia.

57 The attraction of this substrate lies in the fact that scallop shells are made of calcite and
58 therefore the issues associated with aragonite diagenesis and recrystallization to calcite can be
59 circumvented (Penkman et al., 2013). One further advantage of using *Pecten* as a substrate
60 for IcPD dating is that some protein sequence data for this taxon are available and, in future,
61 this could allow in-depth studies of the patterns of protein degradation using a combined
62 approach of mass spectrometry and bulk amino acid analyses (e.g. Demarchi et al., 2013a).

63 The overall aims of our study were to assess if *Pecten* could provide a potentially reliable
64 substrate for IcPD dating and to test the temporal span of the technique in the Mediterranean.
65 Here we present results obtained on both modern (experimental) shells and fossils from
66 raised terraces in the Gulf of Corinth, Greece.

67 As for all studies aimed at establishing the closed-system behaviour of a molluscan species
68 under conditions of natural and accelerated diagenesis, we performed:

- 69 a. Bleaching experiments, to optimise the isolation of an operationally-defined intra-
70 crystalline fraction of proteins.
- 71 b. High-temperature experiments, to test the integrity of the intra-crystalline fraction
72 under continuous leaching conditions and the predictability of the patterns of diagenesis
73 (closed-system behaviour).
- 74 c. Pilot studies on a limited number of fossil shells which were available from
75 geological exposures with good independent age information, to test the suitability of the
76 substrate for dating applications.

77

78 **2. Materials and methods**

79 *2.1 Study site: Gulf of Corinth (Fig. 1)*

80 The Gulf of Corinth (GoC) is an active rift in the eastern Mediterranean (Lat. 38°12'0" N,
81 Long. 22°30'0" E), connected at its western end via a narrow straight to the Ionian Sea. The
82 eastern and southern coastline of the GoC is on the footwall of active normal faults, which
83 (with a small component of regional uplift (see Turner et al., 2010)) results in sediments
84 deposited at the margins of these coastlines being raised to at least 200 m above sea level
85 (asl). Uplift of marine sediments in the eastern side of the Gulf created an isthmus, through
86 which excavations carried out between 1881 and 1893 cut the Corinth Canal, thus exposing
87 extensive Quaternary stratigraphic sections, 5.8 km in height and up to 85 m in height. These
88 include fossiliferous shallow marine facies of Holocene to MIS 11 age (Collier, 1990).
89 Similar facies are particularly well preserved and exposed at intervals around the Perachora
90 Peninsula, which lies north and north-west of the Corinth Canal (Fig. 1). Uplift of marine
91 facies includes *in situ* *Cladocora caespitosa* coral stems, which have been dated by uranium-
92 thorium (U-Th). The age of these corals allowed calculation of uplift rates (e.g. Vita-Finzi
93 and King, 1985; Collier 1990; Dia et al., 1997; Morewood and Roberts, 1999; Leeder et al.,
94 2003, 2005; Roberts et al., 2009; Turner et al., 2010) and provided age control for associated
95 fossil material sampled from the same units.

96 2.2 Sample ages and locations

97 This study used fossil *Pecten* shells collected *in situ*, adjacent to dated coral colonies on the
98 isthmus and Perachora Peninsula (e.g. Fig. 2B). Several coral stems from each location yield
99 U-Th ages of the same marine oxygen isotope stage (MIS) highstand, giving robust age
100 control for the associated *Pecten* shells dated to between MIS 5a/c and at least MIS 11 (Table
101 1). The terminology "MIS ages" is used here to indicate correlated age estimates, obtained by
102 independent dating of the marine oxygen isotope stages. In order to define an aminozone, at
103 least 3 individual shells should be analysed (Miller and Hare, 1980), and therefore this paper
104 only presents a feasibility study on the potential utility of *Pecten*.

105 All *Pecten* shells (identified only to genus level) were collected between 2004 and 2008 by
106 one of us (JT), prior to the IcPD study. *Pecten* and *C. caespitosa* were typically encased in
107 marls with up to 10 m thickness of overlying semi-lithified marine and near-shore sediments
108 (e.g. Fig. 2A). Therefore, although the initial purpose of the shell collection was not for an
109 AAR study, the thickness of the sediments minimises the effect of surface temperature on the
110 rates of diagenesis (Wehmiller, 1977; 1982). The shells were subsequently stored in sealed
111 bags in boxes in rock store room at stable temperatures. Only *in situ* shells that were closely
112 associated with dated coral stems were carefully selected for the IcPD study.

113
114 This contemporaneous fauna lived in the shallow marine zone at depths which typically
115 ranged from 1 to 20 m below sea level, but over time were buried and preserved by marl
116 (calcareous muds and silts) as sediments accumulated on the sea bed. As the coastline
117 prograded and aggraded, shallow marine sediments were overlain by near-shore and then
118 beach deposits (Fig. 2A). Seawards of the beach the sea-bed formed a gentle gradient dipping
119 off-shore; over time marine sediments, including marine fauna, accumulated on the near-
120 shore seabed during the highstand of each MIS warm stage. Where the coastal zone lay on
121 the footwall of active faults, such as the on the Perachora peninsula, these coastal and near-
122 shore deposits were subsequently uplifted. Footwall uplift continued during MIS cold-stages,
123 when eustatic sea level fell; with sea level rise at the subsequent (younger) MIS warm stage,
124 the marine deposits of the previous highstand were now raised above this new sea level. This

125 results in the modern coastal landscape being a flight of MIS warm-stage marine terraces
126 (former sea beds) now at increasing elevation with increased age (Leeder et al., 2003).
127 The terraces with exposures of underlying marine facies are particularly well-preserved on
128 the Perachora Peninsula (Fig. 1C), with palaeo-beaches raised to ~75 m (MIS 7), ~30 m (MIS
129 5e) and ~12 m (MIS 5a/5c) above modern mean sea level (Leeder et al., 2003; Turner et al.,
130 2010). Man-made cuttings through the terraces expose underlying highstand deposits. Some
131 uncover a transition from shallow marine facies to near-shore and then beach sediments as a
132 vertical transition, which records a prograding shoreline (e.g. Fig. 2A). Other exposures
133 (such as at Agriliou Bay) are sea-to-landward cross-sections, revealing an inclined lateral
134 transition of contemporaneous from shallow marine facies to beach facies.

135 Most shells considered here were collected from locations around the Perachora Peninsula,
136 but in order to extend the study beyond MIS 7, we also analysed *Pecten* shells collected from
137 the Corinth Canal (adjacent to corals dated to MIS 9 and 11 (Collier, 1990)). The various
138 sample locations (Fig. 1C) and ages (Table 1) are summarised below:

139 **MIS 11:** A *Pecten* shell Gr 5 was collected from ~70 m elevation on the north side of the
140 Corinth canal (Fig. 2A and B). Associated corals were dated to >350 ka by U-Th (this
141 represented the upper limit of the technique at the time) and correlated with MIS 11 by
142 Collier (1990).

143 **MIS 9:** Shell Gr 6 and associated corals were collected at ~30 m elevation on the north-west
144 side of the canal; the corals were U-Th dated to $311.8 \pm 33.4/-25.8$ ka (MIS 9) by Collier
145 (1990).

146 **MIS 7:** Shell Gr 7 is from the south-east end the Corinth Canal and was collected from ~15
147 m elevation (this location has been down-faulted since MIS 6); corals from this location were
148 U-Th dated to $205.2 \pm 13/-11.7$ ka by Collier (1990).

149 **MIS 5e:** There are several *Pecten* shells from MIS 5e.

- 150 • The corals and associated *Pecten* shell Gr 8 were collected at the 25 m
151 palaeoshoreline of Makrugoaz Ridge. The coral U-Th ages range from 108.5 ± 0.7 to
152 134.0 ± 3.0 ka and were attributed to MIS 5e in Leeder et al. (2003).
- 153 • The corals and associated *Pecten* shell Gr 13 were collected at ~ 23 m elevation, from
154 the New Corinth Terrace. A coral sample was U-Th dated to $132.1 \pm 8.2/-7.5$ ka and
155 correlated with MIS 5e in Leeder et al. (2005).
- 156 • The corals and associated *Pecten* shells Gr 14 and 15 were collected at ~ 10 m
157 elevation at Agriliou Bay. Corals from this location are dated to MIS 5e by Dia et al.
158 (1997) but to MIS 5 to 9 in Roberts et al. (2009). However, a MIS 5e age is
159 considered reliable, as the fossiliferous unit is clearly associated with lateral transition
160 to beach sediments at ~30 m elevation. This is also the back of a marine terrace that is
161 a well-defined topographic feature of the south coast of the Perachora Peninsula and
162 represents uniform uplift of marine terraces on the footwall of an active off-shore
163 fault trending parallel to the coastline (Leeder et al., 2003; Turner et al., 2010). Well-
164 preserved coral samples from other locations beneath the same marine terrace are
165 reliably dated to MIS 5e (Leeder et al., 2005 and references therein). At Agriliou Bay
166 a higher terrace (to ~70 m) is reliably dated to MIS 7, and the lower terrace (~12 m) to
167 MIS 5a/c; thus the range in ages correlating with MIS 5e is attributed to open-system
168 behaviour of these poorly-preserved corals (Turner et al., 2010). *In-situ Pecten* from

169 this marine sequence were sampled from an exposure ~8 m above modern sea level,
170 and overlain by ~4 m semi-lithified near-shore sediments. This location is incised by a
171 river channel which cuts down through the uplifted marine terraces.

- 172 • The corals and associated *Pecten* shells Gr 10 and 11 were collected from 10-12 m
173 elevation at Goat Point, a rubbly terrace of reworked bioherms capping marly
174 sediments with marine fossils. The coral associated with Gr 10 was dated between
175 142.7 +4.6/-4.7 ka and 140.3+4.5/-4.6 ka, which corresponds to late MIS 6/early MIS
176 5e, and independently interpreted as MIS 5e age on stratigraphic relationships by
177 Portman et al., (2005). This terrace can be correlated along the coastline with the ~30
178 m MIS 5e marine terrace beneath which Gr 11 was also sampled.

179
180 **MIS 5a/c:** The corals and associated *Pecten* shell Gr 12 were collected from a 12 m terrace at
181 Australia Gorge, the sample elevation being ~ 6 m above sea level. Coral samples were U-Th
182 dated to 81.9 +1.9/-1.9 ka and thus correlate with MIS 5a/c.

183 **Test shell Gr9 (MIS 5, substage unknown):** The corals and associated *Pecten* shell Gr 9
184 were collected from 6 m elevation at Flagnoro Bay, and whilst the corals yielded a MIS 5a/c
185 age (90.4 +2.3/-2.3 ka; dated by the Open University Uranium-Series Facility (OOUSF);
186 University of East Anglia (UEA) unpublished data), coral from a similar elevation, 110 m to
187 the east, was dated to MIS 5e by Roberts et al. (2009; their sample S2, Table 1).

188 2.3 Sample preparation

189 **A. Fossil shells:** All sub-fossil *Pecten* shells were sampled on the rim to obtain a fragment
190 ~2x5 cm. The shell fragments were first cleaned with a rotary drill to remove any adhering
191 sediment and then sonicated several times in ultrapure water. Dry fragments were crushed to
192 a fine powder (~500 µm), as this size has been found to be optimal for other biominerals
193 investigated for IcPD in the NEaar laboratory (Penkman et al., 2008; Demarchi et al., 2013b),
194 and two samples taken from each powdered fragment. Following the results of the bleaching
195 experiment (Section 3.1), a 48-h bleaching step was used to isolate the intra-crystalline
196 fraction.

197 **B. Modern shells for laboratory experiments:** Five modern *Pecten* sp. shells, bought at a
198 local market in York (UK), were used for bleaching and high-temperature experiments,
199 according to the standard protocol of the NEaar laboratory, described in Penkman et al.
200 (2008). The microstructure of the valves of scallop shells consists of two layers of foliated
201 calcite (Carter, 1989). Here we sampled the rim of each shell in order to select the outer layer
202 only and thus minimise intra-shell variability. The rim of each shell was sampled, cleaned by
203 drilling the periostracum off and sonicating in ultrapure water, and then powdered to ~500
204 µm particle size.

205 **C. Bleaching experiments:** Sequential bleaching experiments were performed by soaking
206 the bulked powders in NaOCl (12% w/v, 50 µL per mg) for various times, in order to assess
207 the effectiveness of bleaching on *Pecten* and determine the optimum time necessary to isolate
208 the intra-crystalline fraction (Penkman et al., 2008; Orem and Kaufman, 2011; Crisp et al.,
209 2013; Demarchi et al., 2013b,c; Tomiak et al., 2013). Five different exposure times to the
210 oxidizing agent were tested (0, 24, 48, 72, 168 h) and three laboratory replicates prepared for
211 each time point (Supplementary Information).

212 **D. High-temperature (kinetic) experiments:** For high-temperature experiments,
213 approximately 20 mg of dry shell powders (bleached for 48 hours) were weighed into
214 individual sterile glass ampoules and 300 μL of ultrapure water added to each sample.
215 According to the NEaar laboratory protocols, no silica glass was added, as the experiments
216 were performed in water and sealed under ambient atmospheric conditions. Three laboratory
217 replicates were prepared for each time point at each temperature. The ampoules were flame-
218 sealed and placed in an oven at 140°C, 110°C or 80°C for various times (Table 2). Water
219 blanks were prepared for selected time points and heated alongside the powder samples
220 (Supplementary Information). After heating, the supernatant water was removed, and the
221 powders left to air-dry before undergoing further preparation for FAA and THAA analysis
222 (section 2.4).

223 **E. Leaching experiments:** For this subset of experiments, the amino acid concentrations
224 detected both in the heated powders and the waters were considered. We compared 48-h
225 bleached and unbleached powders heated at 140°C for 1 hour and 24 hours.

226 2.4 Analysis of intra-crystalline amino acids

227 Each powdered *Pecten* sample was separated into two further subsamples for the analysis of
228 the FAA and THAA fractions. The word “sample” here represents: a) bleached powder of
229 modern shells heated at a certain temperature for a certain time (three laboratory replicates
230 per time point); b) bleached powdered fragments taken from the rim of fossil shell individuals
231 (two per specimen).

232 FAA and THAA subsamples were respectively demineralised in 2 M HCl or hydrolysed in 7
233 M HCl (at 110 °C for 24 hours in nitrogen-flushed vials) and then dried in a centrifugal
234 evaporator according to the standard protocol (Penkman et al., 2008). For leaching
235 experiments, the amino acid concentrations in the water (THAAw) were obtained by
236 removing a 100 μL aliquot of the supernatant water and hydrolysing the peptide bonds in 6M
237 HCl (20 μL per mg/equivalent; Penkman et al., 2008), at 110°C for 24 hours.

238 Overall, we obtained:

- 239 • For bleaching and kinetic experiments: one FAA/THAA pair per laboratory replicate,
240 for a total of three FAA and three THAA subsamples per time point;
- 241 • For leaching experiments: one THAAw subsample per laboratory replicate, for a total
242 of three THAAw subsamples per time point;
- 243 • For fossil *Pecten*, 2 THAA and 2 FAA subsamples per shell specimen.

244 For the analysis of chiral amino acids by reverse-phase liquid chromatography (RP-HPLC),
245 following a modified method of Kaufman and Manley (1998), the extracted amino acids were
246 rehydrated with 10 μL rehydration fluid (containing the non-protein amino acid L-homo-
247 arginine as the internal standard) per mg of original bleached shell powder. Each rehydrated
248 vial was typically analysed once by RP-HPLC; however, a subset of the fossil shell samples
249 was analysed twice (Supplementary Information). Standard solutions of known D/L value
250 and concentrations and procedural blanks were routinely prepared and interspersed in each
251 analytical run. Background levels were calculated by averaging the amino acid concentration
252 detected in the blanks and by calculating the limit of detection $\text{LOD} = \text{Total [THAA]}_{\text{blanks}} \times 3$.

253 During preparative hydrolysis, both asparagine and glutamine undergo rapid irreversible
254 deamination to aspartic acid and glutamic acid, respectively (Hill, 1965), and they are
255 reported together as Asx and Glx. The amino acids reported and discussed in this study are
256 those with optimal chromatographic resolution: Asx, Glx, Gly, Ser, Ala, Val. The amino
257 acids Phe, Leu, Ile are discussed where appropriate, but due to a lack of baseline resolution,
258 they provide only qualitative data.

259 3. Results and Discussion

260 3.1 Characterising the intra-crystalline fraction in Pecten

261 Here we compare the relative amino acid composition of modern *Pecten* shells from our
262 study with that determined in sequenced matrix proteins of scallop shells (*Mizuhopecten*
263 *yessoensis* - *Patinopecten yessoensis*): MSP-1 and SP-S (Sarashina and Endo, 1998, 2001;
264 Hasegawa and Uchiyama, 2005) and the two nacrein-like proteins P1 and P2 (Norizuki and
265 Samata, 2008), which act as a negative regulator of calcification in the shells of molluscs.
266 Cys, Lys, Pro and Trp are not routinely quantified with our RP-HPLC method; Tyr is
267 observed but was not quantified for this study, while Met degrades (oxidises) during
268 preparation, therefore we adjusted the theoretical composition of the matrix proteins
269 accordingly. We note that the composition of unbleached *Pecten* (Fig. 3A) is similar to that
270 of MSP-1 and SP-S, with high relative proportions of Ser, Gly, Asx, Ala and Glx (Fig. 3C
271 and 3D). The percentage values differ (e.g. Asx represent 24% and 28% of the composition
272 of MSP-1 and SP-S, and 32% in *Pecten*), possibly because the measure of bulk amino acids
273 from a fraction of proteins of unknown sequence might not be the same as the theoretical
274 composition of known sequences; however, the overall proportion of Asx, Ser and Gly are
275 broadly equivalent in MSP-1, SP-S, and unbleached *Pecten*. On the contrary, the nacrein-like
276 proteins P1 and P2 have higher relative levels of the acidic amino acids Asx and Glx
277 compared to Ala, Gly and Ser (Figs 3E and 3F).

278 Bleaching has a significant effect on the matrix proteins retained in *Pecten*: after 48 h
279 exposure to NaOCl, only 6% of the original THAA are retained (Fig. 4A), a value that
280 remains stable after prolonged bleaching. Interestingly, the relative percentage of Ser and Gly
281 decreased in bleached shells compared to unbleached powders, although still dominated by
282 Asx, Glx, Ser and Gly (Fig. 3B). This may indicate that Asx and Glx-rich nacrein-like
283 proteins dominate the intra-crystalline pool, while Ser and Gly-rich MSP and SP proteins are
284 mainly inter-crystalline. The comparison suggests that two very different pools of proteins
285 characterise the intra- and inter-crystalline environments in *Pecten*. The Asx- and Glx-rich
286 fraction may even offer superior performance as a substrate for geochronology over
287 Quaternary timescales rather than the Ser-rich pool, as Ser diagenesis is complicated by
288 decomposition and a reversal in D/L values (section 3.3). Further, the knowledge that
289 nacrein-like proteins are associated with the (operational) intra-crystalline fraction supports
290 the hypothesis that these play an important role in biomineralisation itself (Norizuki and
291 Samata, 2008).

292 The extent of bleach-induced racemization is negligible between 24 and 72 hours (< 0.03 D/L
293 units for Asx; Figure 4B and Supplementary Information), although after 72 h bleaching a
294 further increase is observed for Asx and Ser, thus suggesting that longer bleaching times may
295 compromise the integrity of the carbonate phase. Therefore we chose a 48-h bleaching step
296 (standard for the NEaar laboratory) for all further analyses on *Pecten*. The percentage of

297 amino acids removed by 48-h bleaching in *Pecten* is ~95%, higher than for other bleached
298 marine molluscs (e.g. 85-90% in *Patella*, 30% in *Glycymeris* (Demarchi et al., 2013b, 2015;
299 Ortiz et al., 2015)). The average value of total THAA concentration in 48-h bleached *Pecten*
300 powders was ~2.6 nmol/mg, similar to 48-h bleached *Patella* (~2.8 nmol/mg) and higher than
301 bleached *Glycymeris* (~1.5 nmol/mg) (Demarchi et al., 2013b, 2015).

302 These results provide evidence for the presence of an operationally defined intra-crystalline
303 fraction of proteins that can be isolated by a 48-h bleaching step, similar to previous
304 observations for other carbonate biominerals (Penkman et al., 2008; Hendy et al., 2012;
305 Demarchi et al., 2013b).

306 3.2 Leaching experiments: test of closed-system behaviour

307 Closed-system tests are used to assess the stability of the intra-crystalline fraction upon
308 conditions of continuous leaching in the laboratory, accelerated by high temperatures. In the
309 NEaar laboratory, heating experiments are typically conducted on a homogenised “bulk”
310 sample of bleached powders from modern shells (section 2.3) in order to determine the extent
311 of any leaching from the system. However, it is important to note that heating bleached
312 powders might not accurately mimic the conditions in the natural environment. Therefore,
313 while our approach provides a useful proxy to compare the behaviour of unbleached and
314 bleached shell powders, we stress that results presented on bleached shells here (and in other
315 studies, e.g. Penkman et al., 2008; Demarchi et al., 2105) may not be comparable to data
316 obtained by other authors on unbleached material (e.g. Hare and Mitterer, 1969; Miller et al
317 2000; Kaufman, 2006; Ortiz et al., 2015).

318 Results on bleached and unbleached *Pecten* powders show that significant leaching of amino
319 acids into the water occurred for the unbleached material (21% of the overall amino acid
320 concentration after 24 h heating). The amino acid concentration detected in the water from
321 the bleached samples was lower than background levels (LOD) (Fig. 5A) and represents ~0.
322 4% after 24 h heating (Table 3). While this may indicate that the system (heated bleached
323 shells) is not perfectly closed, the high levels of leaching from unbleached shells support the
324 hypothesis that the isolation of the intra-crystalline fraction in fossil *Pecten* may be important
325 for geochronological studies.

326 It is important to note that the isolation of the intra-crystalline fraction results in major
327 differences in racemization values. Figs. 5B-C-D-E display THAA D/L values detected in
328 bleached and unbleached shell powders heated for 1 and 24 hours at 140°C: despite the
329 limited dataset (two time-points only), the intra-crystalline fraction is significantly more
330 racemized (up to 0.5 D/L units difference) than its unbleached (“whole-shell”), counterpart,
331 indicating that the pathways of early diagenesis in bleached and unbleached shells diverge.
332 Therefore, patterns of IcPD (i.e. on bleached shells – section 3.3) will not necessarily mimic
333 patterns observed when unbleached shells are analysed.

334 3.3 Patterns of IcPD at high and low temperatures

335 The results of bleaching and leaching experiments show that bleached modern *Pecten*
336 samples yield a fraction of intra-crystalline amino acids which is not leached under
337 conditions of prolonged artificial diagenesis (Figs 4 and 5A). Furthermore, high-temperature
338 experiments demonstrate that the patterns of hydrolysis, decomposition and racemization are
339 predictable: Fig. 6 shows that the %FAA (i.e.: $[\text{FAA}]/[\text{THAA}] \times 100$) and D/L values increase

340 steadily with heating time, although %FAA decrease for the last time-point (120 h),
341 presumably due to decomposition (140 °C data in Fig. 6; 110 °C and 80 °C data in
342 Supplementary Information). A well-known exception to the increase in THAA D/Ls over
343 time is represented by Ser, which displays a reversal (i.e. in *Pecten* D/Ls increase to ~0.5 and
344 then decrease). In addition [Ser] values fall below the LOD in bleached *Pecten* heated at
345 140°C for 2 hours. Nonetheless, the initial diagenesis of Ser and the patterns of degradation
346 of the fast-racemizing Asx can be useful as a geochronometer for shorter timescales (e.g.
347 Demarchi et al., 2011; Hendy et al., 2012), while slow-racemizing residues (Glx, Val) could
348 be useful for older material. The very high levels of racemization of Ala (usually a medium-
349 rate racemizer), may be affected by the rapid decomposition of Ser, thus resulting in higher-
350 than-expected DL ratios: in the 140°C experiment, THAA Ser concentrations fall from ~400
351 to ~100 pmol/mg between unheated and 1-h heated bleached samples. In the same time
352 interval, THAA Ala concentrations increase by ~60 pmol/mg, therefore accounting for less
353 than one third of the loss in THAA Ser. The increase in THAA Ala D/Ls may also be due to
354 the positions of Ala in the protein chain (e.g. if a significant proportion was next to a rapidly-
355 hydrolysed peptide bond, resulting in enhanced racemization of Ala in an N-terminal position
356 (Mitterer and Kriaušakul, 1984)).

357 The predictable patterns of diagenesis displayed by bleached *Pecten* heated at high
358 temperatures suggest that *Pecten* has very good potential as a substrate for IcPD
359 geochronology. However, diagenesis at high temperature may differ significantly from the
360 breakdown pathways occurring at the normal burial temperature, as we have reported for a
361 range of biominerals, including marine mollusc shells and corals (Demarchi et al., 2013c;
362 Tomiak et al., 2013). “Natural” diagenesis of bleached subfossil *Pecten* appears to behave in
363 a way that resembles closely the high-temperature patterns with regards to racemization
364 (FAA D/L vs THAA D/L plots, Figs 7A, 7C, 7E, 7G). On the contrary, %FAA vs THAA D/L
365 plots (Figs 7B, 7D, 7F, 7H) show that, for the same THAA D/L, bleached shell powders
366 heated at high temperatures yielded lower %FAA values than fossil bleached shells. We
367 observed a similar pattern in bleached *Patella* (Demarchi et al., 2013c) and interpreted it as
368 indicating that the activation energies for peptide bond hydrolysis were lower than those for
369 racemization, which implies that at lower temperatures hydrolysis will be more facile than
370 racemization. We cautiously suggest a similar interpretation here, which however will
371 require further testing on a more extensive set of high-temperature experiments on both
372 unbleached and bleached *Pecten*. We also note that the higher variability displayed by fossil
373 data may be partly due to the localised burial/thermal environment of each shell, but also
374 natural inter-shell variability in protein composition in individual shells, compared to the
375 homogenised powders used for kinetic experiments.

376 The variability of the fossil data is lower in THAA vs FAA D/Ls plots than in THAA D/Ls vs
377 %FAA plots and the trajectory of co-variance is coherent (from less degraded to most
378 degraded), thus indicating good closed-system behaviour. The only exception is represented
379 by FAA D/L values for Glx, but this is likely to be due to the preferential formation of FAA
380 Glx as a lactam (e.g. Walton, 1998), which cannot be detected with our analytical method.

381 However, as kinetic experiments were conducted on powders bleached pre-heating rather
382 than post-heating, the patterns observed here might not mimic the pathways of diagenesis
383 occurring in the natural environment. An example of these dissimilarities is highlighted by
384 the difference in total amino acid concentrations recovered from fossil and modern shells.

385 The sum of Asx, Glx, Ser, Ala, Gly and Val THAA concentrations found in bleached fossil
386 shells of MIS 5 age (~3.5 nmol/mg, $\pm 12\%$) is higher than that found in bleached modern shell
387 powders (~2.6 nmol/mg). MIS 7 and MIS 9 shells also yielded slightly higher values (~2.8
388 and 3.2 nmol/mg, respectively), while the concentrations in the MIS 11 specimen were lower
389 (~1.8 nmol/mg) (Supplementary Information). Higher-than-expected concentrations in the
390 fossils indicate that diagenesis may result in matrix-protein interactions which may not be
391 disrupted by bleaching, resulting in the isolation of a different fraction of organics. This does
392 not preclude that the fraction isolated from fossil shells appear to behave as a closed system:
393 the covariance of FAA vs THAA D/L values (Fig. 7) shows that open-system behaviour does
394 not seem to affect the shells analysed as part of this study.

395 These results indicate that whilst *Pecten* is a shell taxon that should be targeted for IcPD
396 dating, the patterns of diagenesis are (unsurprisingly) as complex as those found in other
397 biominerals, particularly regarding the temperature sensitivity of the different breakdown
398 reactions. We have not used the high temperature kinetic data to extrapolate reaction rate
399 parameters to burial temperatures, but larger fossil datasets should enable kinetic modelling
400 in future studies.

401 *3.4 Using bleached Pecten as a geochronological indicator for the Quaternary of the* 402 *Mediterranean*

403 Calibration of the extent of IcPD with independent numerical dating methods is especially
404 important over Quaternary timescales encompassing multiple glacial-interglacial cycles (see
405 Wehmiller et al., 2012) and in sedimentary deposits in mid-latitude areas with strong
406 temperature gradients, such as the Mediterranean. In this study, we tested the feasibility of
407 using bleached *Pecten* for aminostratigraphy, using nine bleached *Pecten* shells from seven
408 Pleistocene raised beaches, located in the area of the Corinth Gulf, dated by U-Th and
409 ranging from 81 ka to > 350 ka (Table 1). The aim of this preliminary work was to assess
410 whether the protein diagenesis in the intra-crystalline fraction of subfossil *Pecten* showed a
411 consistent pattern with age, the potential levels of resolution and to establish the temporal
412 span of the technique on this substrate in this geographic area.

413 Applying statistical analyses to AAR data is complicated by the fact that D/L values have
414 upper and lower boundaries, and the small sample sizes in this study limit the confidence in
415 the statistical results, but the data were interrogated using ANOVA and Bonferroni-Holm
416 tests. Here, the number of shell samples for MIS5 is much higher than for other stages; the
417 results of the Bonferroni-Holm test (Supplementary Information) should therefore be viewed
418 with caution. Despite these limitations we report the results of the statistical tests to provide
419 preliminary indication on the suitability of *Pecten* as a substrate for IcPD for age estimation.

420 Asx THAA D/Ls (Fig. 8A, note scale) are still relatively far from equilibrium (D/L=1) and
421 are able to provide well-resolved age differences between shells belonging to different MISs:
422 significant differences are found between MIS 5 / MIS 9 and MIS 5 / MIS 11, but not
423 between MIS 5 / MIS 7, MIS 7 / MIS 9 or MIS 9 / MIS 11, although a general increase of
424 D/L values with age can be observed (Fig. 8A). Asx MIS 11 values (Gr 5) are lower than
425 expected (similar to MIS 7 values), and this has an impact on the age resolution. Asx is the
426 only amino acid displaying this pattern for Gr5, but concentrations for this shell are lower
427 than for other shells analysed in this study (Supplementary Information); we therefore
428 hypothesise that this result might be due to decomposition of the highly-racemized FAA Asx

429 fraction., Any within-MIS 5 differences could not be resolved, and the test shell Gr9 did not
430 show significant differences with either MIS 5e or MIS 5a/c (or MIS 7) shells.

431 The amino acid Ala (Fig. 8E), usually a medium-rate racemizer, displays D/L values between
432 0.45-0.85, higher than those of the fast racemizer Asx, as already shown by the high-
433 temperature dataset (Fig. 6). The rapid decomposition of Ser into Ala (observed in the kinetic
434 experiments) indicates that the patterns observed may reflect the interplay of decomposition
435 and racemization of both amino acids. Ala THAA D/Ls for MIS 5 are significantly different
436 from MISs 7, 9, 11 but MISs 7, 9 and 11 cannot be resolved (Bonferroni-Holm results in
437 Supplementary Information). The level of variability within MIS 5e, although not
438 exceptionally high (CVs ~9%) is largely due to Gr13, which falls at slightly lower D/L values
439 than other MIS 5e shells. THAA [Ser]/[Ala] values (Fig. 8D) might reflect directly the
440 decomposition of Ser into Ala, but the only statistically significant difference is found
441 between Gr9 and MIS 7 shells. These results therefore strengthen the conclusion that Gr9 is
442 MIS 5 in age, although the sub-stage within MIS 5 cannot be better resolved.

443 Slow-racemizing amino acids (Glx and Val; Figs 8B and 8C) should represent the best
444 indicators of diagenesis over Pleistocene timescales; however, in bleached *Pecten* both amino
445 acids display high D/Ls and therefore this limits their usefulness for older shells (here, MIS
446 7-11). Glx THAA D/Ls are able to resolve age differences between shells of MIS 5 and MISs
447 7/9/11 age; shells Gr9 (test sample) and Gr12 (MIS 5a/c) are also significantly different, thus
448 supporting an attribution of Gr9 to an earlier substage within MIS 5. Val THAA D/Ls
449 perform slightly worse than Glx D/Ls over younger timescales, with both MIS 5 values
450 indistinguishable from MIS 7 D/Ls, although significantly lower than MIS 9 and MIS 11.

451 4. Conclusions

452 The extent of protein degradation in modern and sub-fossil *Pecten* shells was investigated
453 through the concurrent use of the closed system approach of Penkman et al. (2008) and the
454 analysis of multiple amino acids (Kaufman and Manley, 1998). When tested against the
455 raised beaches located in the area of the Corinth Gulf, the IcPD in subfossil *Pecten* showed a
456 consistent diagenetic pattern with age. Our results showed that:

- 457 • A 48-h bleaching step is effective in isolating the intra-crystalline fraction of proteins
458 shells of the modern marine mollusc *Pecten* (Fig. 4), similar to that observed for other
459 mollusc shells (Sykes et al., 1995; Penkman et al., 2008; Demarchi et al., 2013b).
- 460 • The intra-crystalline proteins are likely to belong to the group of nacrein-like proteins
461 on the basis of their amino acid composition (Fig. 3).
- 462 • The minimal loss of amino acids from the bleached shell during high temperature
463 (leaching) experiments supports the evidence for the system's integrity (Fig. 5), and
464 we suggest that the intra-crystalline fraction of *Pecten* shells better approximated a
465 closed system than the unbleached fraction.
- 466 • *Pecten* shows good potential for retaining an effectively closed system of proteins
467 over geological timescales (Fig. 7), therefore it can be identified as a useful substrate
468 for future IcPD geochronological studies.
- 469 • The diagenetic patterns followed by fossil shells and modern shells heated at high
470 temperature were similar for the amino acids Asx, Glx, Ala and Val, but only when
471 the co-variance of FAA vs THAA D/Ls were considered, whilst plots of THAA D/Ls

472 vs %FAA showed significant differences between the reactions at high and low
473 temperatures (Figs 6, 7).

- 474 • The small sample sizes in this preliminary study preclude a full assessment of the
475 aminostratigraphic power of *Pecten* IcPD, but this pilot study shows that the resolution
476 of IcPD data on *Pecten* for the Gulf of Corinth is potentially sufficient to distinguish
477 between shells of MIS 5 age from older material. It was not possible to differentiate
478 the single specimens analysed from MIS 7, 9 and 11 deposits in this study, although
479 we observed a coherent increase in Ala, Glx and Val D/L values and decrease in
480 [Ser]/[Ala] with age between MIS 7 and MIS 11.
- 481 • Val and Ala D/L values approach equilibrium for MIS 11 shells and this results in
482 loss of resolution for shells older than 350 ka. However, Glx is further from
483 equilibrium for shells of this age, and may allow the limits of the technique to be
484 extended beyond MIS 11 in the Mediterranean area.
- 485 • The analysis of a larger number of fossil shells in future studies will allow the
486 assessment of the within-MIS natural variability and the level of resolution to be
487 refined.

488

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624 **Tables**

625 **Table 1:** Details of fossil *Pecten* sp. shells from raised beach deposits of the Corinth Gulf,
 626 Greece, analysed for IcPD dating. Numerical ages (ka) were obtained by U/Th dating (TIMS)
 627 on *in-situ* coral samples. ¹(Collier, 1990); ²(Leeder et al., 2003); ³(UEA samples dated by
 628 OUUSF); ⁴(Leeder et al., 2005); ⁵(Dia et al., 1997).

Sample name	Site name	Control U/Th Age (ka)	Marine Isotope Stages	Subsamples per shell (n)	NEaar numbers
Gr5	Corinth Canal	>350	¹ MIS 11	2	6856/6857
Gr6	Corinth Canal	311.8 +33.4/-25.8	¹ MIS 9	2	6858/6859
Gr7	Corinth Canal	205.2 +13.0/-11.7	¹ MIS 7	2	6860/6861
Gr8	Makrugoaz Ridge	108.5±0.7 to 134.0±32.0	² MIS 5e	2	6862/6863
Gr9	Flagnoro Bay	90.4+2.3/-2.3	³ MIS 5	2	6864/6865
Gr10	Goat Point	142.6+4.6/-4.7 - 140.3+4.5/-4.6	³ MIS 5e	2	6866/6867
Gr11	Goat Point	stratigraphic correlation	MIS 5e	2	6868/6869
Gr12	Australia Gorge	81.9+1.9/-1.9	MIS 5a/c	2	6870/6871
Gr13	New Corinth Terrace	132.0+8.2/-7.5	⁴ MIS 5e	2	6872/6873
Gr14	Agriliou Bay	Between 123.1±0.7 and 120.5±0.9	⁵ MIS 5e	1	6874
Gr15	Agriliou Bay	Between 123.1±0.7 and 120.5±0.9	⁵ MIS 5e	2	6876/6877

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634 **Table 2.** Temperatures and time points used for kinetic experiments on bleached *Pecten*
 635 shell. * unbleached samples were also prepared for these time points. Water samples were
 636 analysed for bleached and unbleached powers heated for 1 and 24 hours at 140°C.

Temperature (°C)	Time points (h)						
140	1*	2	4	8	24*	48	120
110	24	120	240	481	865	1200	
80	24	99	120	720	1200		

637

638 **Table 3.** % loss of amino acids from unbleached and bleached *Pecten* powders after 24 h of
 639 heating at 140 °C (n = number of samples). Total concentrations were calculated using Asx,
 640 Glx, Ser, Gly, Ala and Val. The average blank total concentration (147 pmol/mg) was
 641 subtracted from all values.

Loss of amino acids after 24 h heating at 140 °C	Unbleached	Bleached
Initial [total] THAA concentration in shell, unheated (pmol/mg) (n = 3)	52757	2366
[total] THAA after heating (pmol/mg) for 24 h	5850	1580
[total] THAA _w in water, heated (pmol/mg equiv.)	11059	10
Overall loss in shell (%)	89%	33%
Loss into water, by leaching (%)	21%	0.4%
Loss by decomposition (%)	68%	33%

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654 **Figure Legends**

655 **Figure 1.** Sampling location in the eastern Mediterranean (A): the Perachora Peninsula and
656 Corinth Canal in the Gulf of Corinth, Central Greece (B). C: Location of *Pecten* and coral
657 samples from around the uplifted coastline.

658 **Figure 2.** During each marine highstand the marine sediments aggrade and prograde,
659 forming a sequence of sediments associated with each highstand which typically coarsen up-
660 section, from off-shore marine to shallow marine to beach facies (A). As the region has been
661 uplifting since at least MIS 11, these sediments are raised above sea level with well-preserved
662 fossil material. The white square in Fig. 2A (enlarged in Fig. 2B) shows the sample location
663 for Gr 5 (MIS 11). C: *Pecten jacobaeus*.

664 **Figure 3.** THAA composition of unbleached (A) and 48-h bleached (B) *Pecten* shells.
665 Sequenced matrix proteins of *Mizuhopecten yessoensis* (*Patinopecten yessoensis*): Scallop
666 shell matrix protein MSP-1 (C); Scallop shell matrix protein SP-S (Sarashina and Endo,
667 1998, 2001; Hasegawa and Uchiyama, 2005) (D); Nacrein-like protein P2 (E); Nacrein-like
668 protein P1 (Norizuki and Samata, 2008) (F).

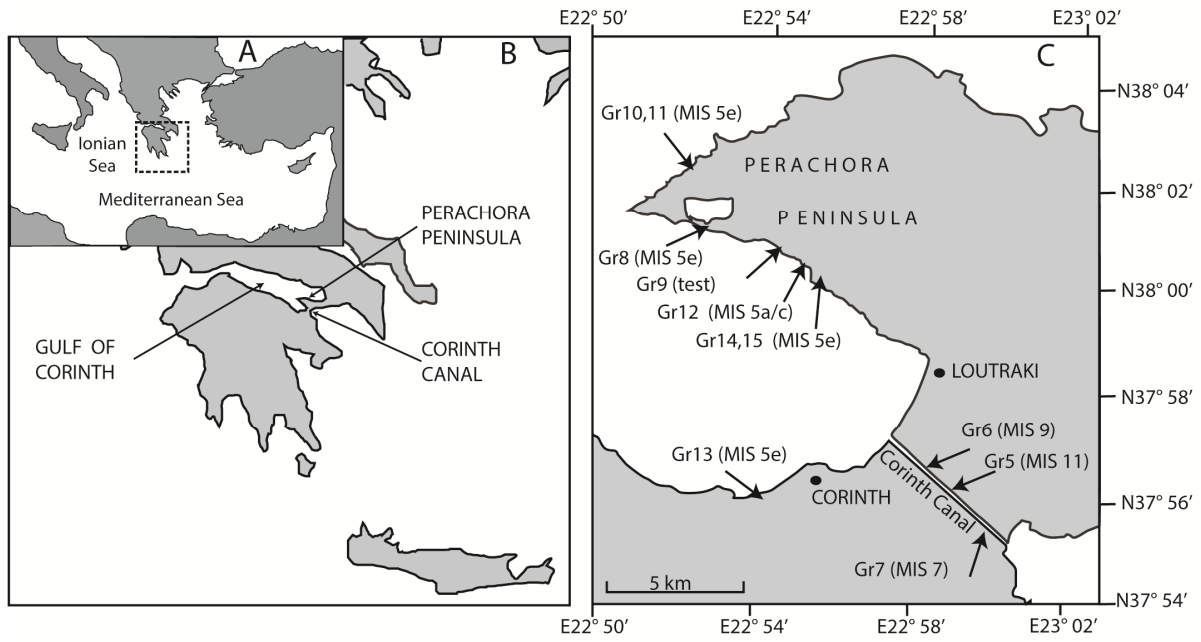
669 **Figure 4.** A: Decrease in THAA concentration with increasing bleaching time for modern
670 *Pecten* (bulk powders). B: Effect of bleaching on Asx THAA D/L values in modern *Pecten*
671 shells. Error bars (uncertainty contained within symbols) represent one standard deviation
672 around the mean, calculated on three laboratory replicates for the same time point.

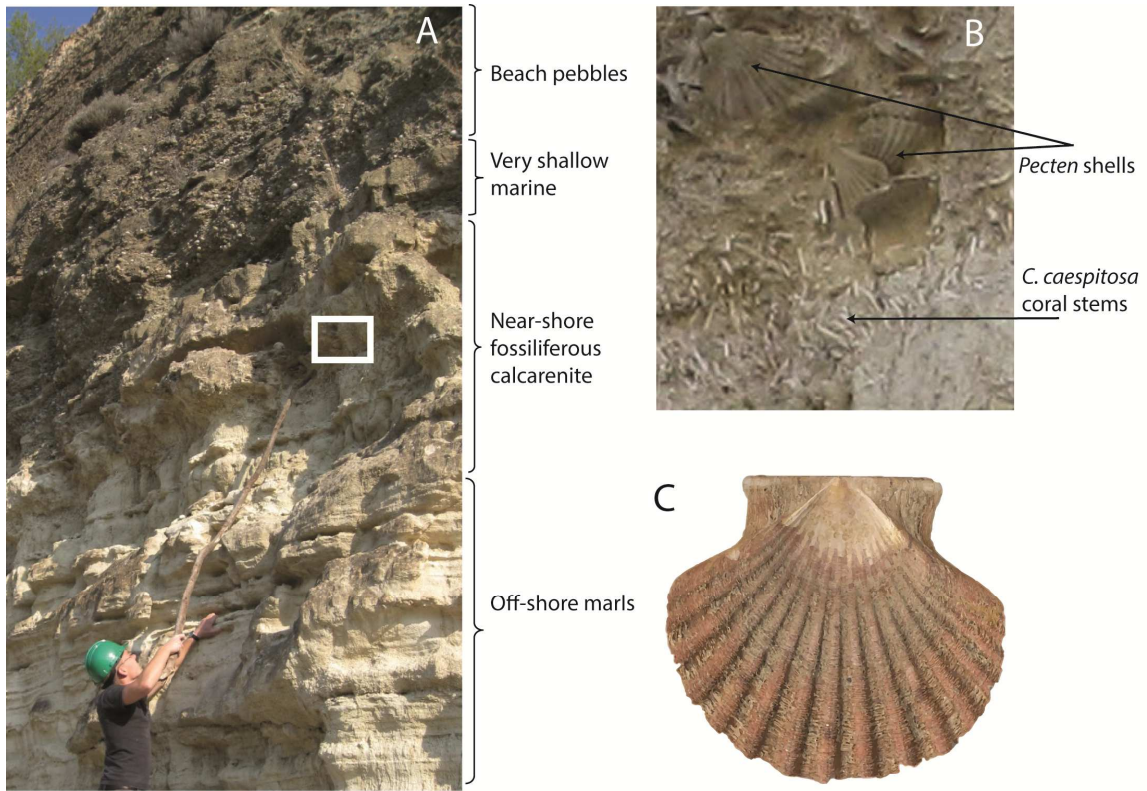
673 **Figure 5.** A: Limit of detection (LOD) compared to the concentrations of amino acids in the
674 water used to heat (140°C for 24 hours) bleached and unbleached *Pecten*. Error bars represent
675 one standard deviation around the mean, calculated on three laboratory replicates for the
676 same time point. B, C, D, E: THAA D/L of Asx (B), Glx (C), Ala (D), Val (E) detected in
677 bleached and unbleached modern *Pecten* heated at 140°C for 1 and 2 hours.

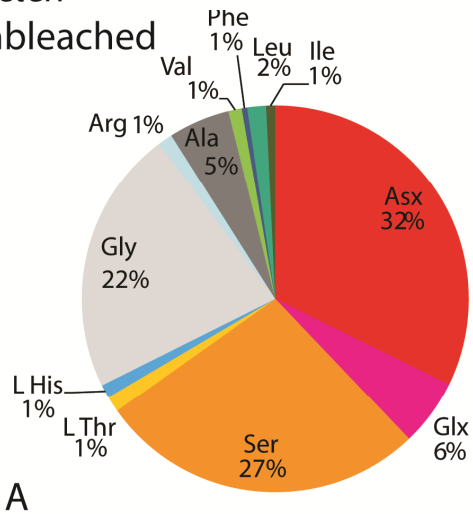
678 **Figure 6:** THAA D/L (A) and %FAA (B) values measured on bleached *Pecten* powders
679 heated at 140°C. [Ser] values fall below LOD after 2 hours heating and therefore %FAA Ser
680 were not calculated, although all time points are shown in Figure 6A. Error bars represent one
681 standard deviation around the mean, calculated on three laboratory replicates for the same
682 time point.

683 **Figure 7:** Patterns of diagenesis at high and low temperatures in bleached modern and fossil
684 *Pecten* powders. THAA vs FAA D/L plots for Asx (A), Glx (C), Ala (E) and Val (G); THAA
685 D/L vs %FAA plots for Asx (B), Glx (D), Ala (F) and Val (H). Val values for the 80°C
686 experimental samples were not determined as the concentrations of FAA D-Val were too low
687 to be detected.

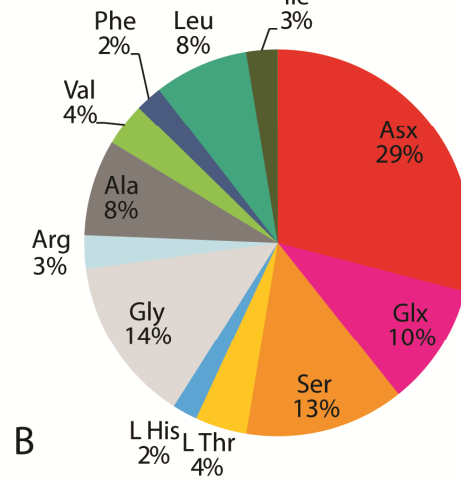
688 **Figure 8:** THAA D/Ls for Asx (A), Val (B), Glx (C) and Ala (E) and THAA [Ser]/[Ala]
689 values (D, note that y-axis values are plotted in reverse order) for fossil bleached *Pecten*
690 shells from geological deposits of known age (U-Th dates, Table 1), Gulf of Corinth, Greece.
691 Vertical error bars represent the expected coefficient of variation (CV) for each amino acid,
692 calculated on the basis of the securely-dated MIS 5e shells. Horizontal error bars represent
693 the U-Th numerical age error (Table 1). Test shell Gr9 (dark grey squares) is plotted in the
694 mid-point of MIS 5, with horizontal error bars spanning the whole of MIS 5.





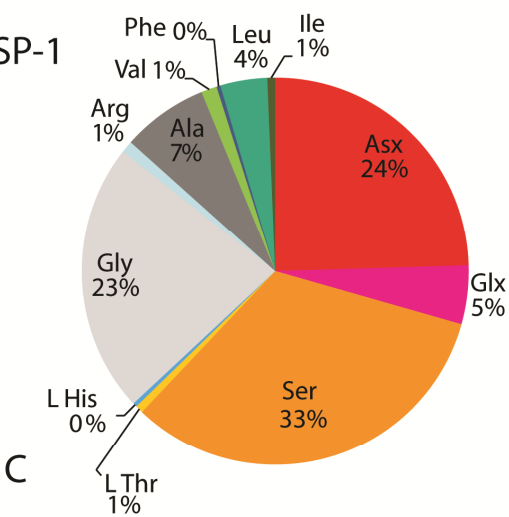
Pecten
unbleached

A

Pecten
bleached

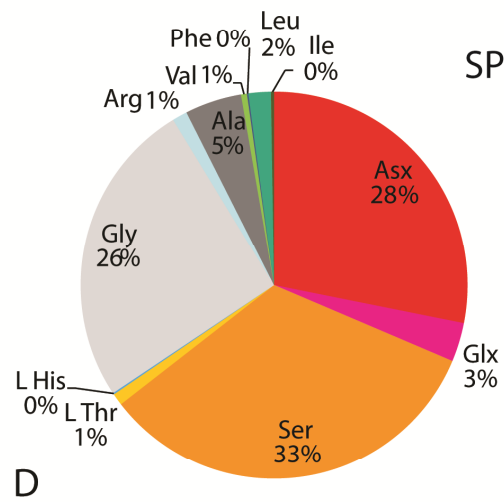
B

MSP-1



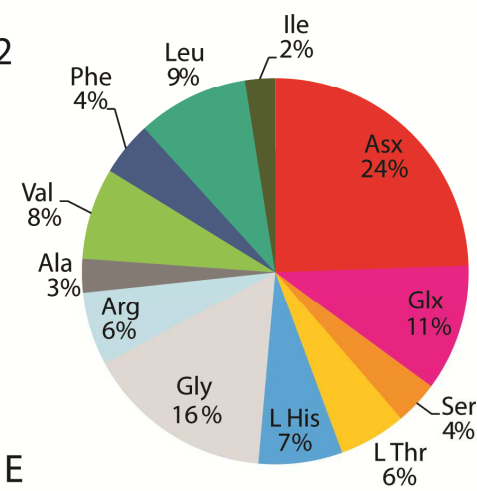
C

SP-S



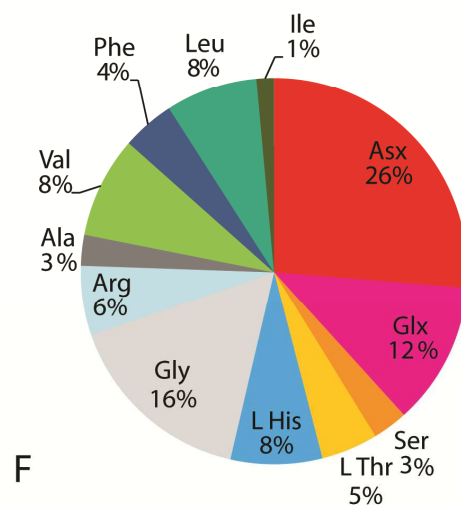
D

P-2



E

P-1



F

