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1 Acid-induced demineralisation of human enamel as a function of time and pH
2 observed using X-ray and polarised light imaging

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23 **Abstract**

24

25 Acid-induced enamel demineralisation affects many individuals either by exposure to
26 acidic diets, acidic gas pollution (dental erosion) or to dental plaque acids (dental caries).

27 This study aimed to develop *in situ* X-ray and light imaging methods to determine
28 progression of enamel demineralisation and the dynamic relationship between acid pH and
29 mineral density.

30 Hourly digital microradiograph time-lapse sequences showed the depth of enamel
31 demineralisation in 500 μm thick sections progressed with time from the surface towards the
32 dentine following a power-law function, which was 21% faster than the lateral
33 demineralisation progression after exposure for 85 h to lactic acid (10%, pH 2.2). The
34 minimum greyscale remaining (mineral content) within the induced enamel lesion followed
35 an exponential decay, while the accumulated total greyscale loss with time was linear, which
36 showed a constant anisotropic mineral release within the enamel architecture. This 85h
37 demineralisation method studied by polarised light microscopy time-lapse sequences showed
38 that once the demineralisation front reached the enamel Hunter-Schreger bands, there was
39 preferential demineralisation along those bands.

40 Mineral density loss was linear with increasing pH acidity between pH 5.2 and pH 4.0
41 (with 0.4 pH increments) when incubated over a 3-week period exposed to 0.5% lactic acid.
42 At pH 4.0, there was complete mineral loss in the centre of the demineralised area after the 3-
43 week period and the linear function intercepted the x-axis at \sim pH 5.5, near the critical pH for
44 hydroxyapatite (HAp). These observations showed how intrinsic enamel structure and pH
45 affected the progression of demineralisation.

46

47 **Keywords:** Enamel; demineralisation; imaging; *in situ*; kinetics.

48 **1. Introduction**

49 Dental caries is the most prevalent disease in modern humans [1], [2], [3] and
50 considerable efforts are made to promote good oral hygiene by routine mechanical removal
51 of plaque (brushing with dentifrices), use of antimicrobial and fluoridated agents and the
52 avoidance of a high sugar diet [4]. However, the high prevalence of the disease indicates that
53 current methods and products are not able to completely prevent it. This paper examines the
54 possible role of intrinsic enamel structure factors and pH in dental caries progression in order
55 to gain understanding of the dynamics of the demineralisation process and aid in the
56 development of improved prevention, diagnosis and restorative treatments [5], [6], [7].
57 Theoretical mathematical models of caries initial propagation and progression have been
58 proposed but still require experimental validation [8], [9].

59 Enamel, the outer most layer of teeth, is the hardest tissue in the human body,
60 consisting of 96% hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) (HAp), 3% water and 1% organic
61 material [10]. Enamel demineralisation is initiated by exposure to acidic conditions, causing
62 hydroxyapatite dissolution. Whilst the critical pH at which this occurs depends on a variety of
63 factors, it is generally accepted to be around pH 5.5 [11]. Dental caries is associated with
64 acidogenic bacteria forming sticky biofilms that rapidly metabolise carbohydrates and excrete
65 acids *e.g.* lactic acid [12]. The microbiological element of dental caries is fundamental to the
66 disease, but there are numerous variables, which can affect cariogenicity *e.g.* bacterial
67 species, biofilm composition, saliva composition, as well as quantity, type and frequency of
68 carbohydrate intake in diet. Since this study aimed to characterise the process of acid
69 demineralisation of enamel, an acid-only caries model was used.

70 Bulk enamel has a prismatic structure consisting of 5 μm sized HAp rods (with a
71 small amount of organic material remaining after enamel mineralisation and maturation

72 processes) containing crystallites orientated with long axes that run parallel to the
73 longitudinal axis of the rod and HAp inter-rods which contain crystallites that laterally flare
74 until orientated approximately perpendicular to the rods [13]. Acid-induced enamel
75 demineralisation has shown preferential rod dissolution patterns (type 1), preferential inter-
76 rod dissolution (type 2) and a mixture of both (type 3) when imaged using scanning electron
77 microscopy (SEM) [14], [15]. This anisotropic HAp dissolution has also been observed in
78 small and wide angle X-ray scattering synchrotron experiments [16]. The demineralisation
79 pattern in enamel caries is initially type 2 but as lesions deepen type 1 demineralisation
80 becomes more prevalent [17]. However, SEM observations have focused on *ex-situ* prism
81 scale changes and have not taken into account *in situ* kinetics or the schmelzmuster
82 anisotropy, *e.g.* radial enamel (parallel prism arrangement) and Hunter-Schreger bands
83 (HSBs) (bundles of parallel prisms with alternating tilt angles (decussating)) [18], [19], which
84 could affect the kinetics of acid front advance.

85 Radiographic longitudinal studies of human enamel demineralisation has the potential
86 to overcome these issues [20] but has not yet harnessed the digital microradiographic imaging
87 capability of microcomputed tomography (micro-CT) equipment which can be used for
88 automated *in situ* 2D imaging, enabling more time points to be captured with high image
89 resolution. Light microscopy imaging of longitudinal tooth sections can also detect enamel
90 demineralisation. Early stage carious lesions in enamel (without cavitation), are typically
91 organised into four or five zones according to refraction patterns of light. From the tooth
92 surface towards the depth of the enamel these zones are referred to as the surface zone, the
93 body of the lesion, the dark zone and translucent zone. An extra translucent zone is
94 sometimes seen between the dark zone and the body of the lesion. All of these zones are
95 thought to represent changes in enamel porosity and crystallite content [21] that result in
96 altered and localised refraction properties of enamel. However, this technique has not been

97 fully utilised to image the demineralisation process *in situ* nor been compared with an *in-situ*
98 digital microradiography technique.

99 To fully characterise the demineralisation process other factors need attention,
100 including the pH of the environment in contact with enamel. It is well established that as pH
101 decreases, the severity of enamel demineralisation increases [22] but the relation between the
102 two has not been precisely established to date. Therefore, the aim of this work was to assess
103 enamel demineralisation as a function of time and pH as well as elucidating how
104 demineralisation related to the intrinsic properties of enamel. Automated time-lapse
105 experiments were established to monitor the *in situ* enamel demineralisation by lactic acid,
106 using digital microradiography and polarised transmitted light microscopy (PTLM) to
107 determine the kinetics of the demineralisation advance. Comparison between
108 demineralisation detected by digital microradiography and PTLM was made to establish any
109 differences in sensitivity of the two techniques. The mineral density change in relation to
110 lactic acid pH as well as assessing the shape of demineralised lesions that resulted was
111 established using micro-CT 3D reconstructions.

112 **2. Materials & Methods**

113 **2.1 Sample preparation**

114 The study used intact human third molars extracted for non-carries related therapeutic
115 reasons (National Research Ethics Committee; NHS-REC reference 09.H0405.33/
116 Consortium Reference BCHCDent332.1531.TB). Tooth root tips were removed using a low
117 speed rotating diamond saw (Isomet, UK) before sterilisation in 10% formalin buffered
118 solution (Sigma Aldrich, UK) for four days. Teeth were then rinsed with water and sectioned
119 into 500 µm thick slices from the buccal to the lingual surfaces, the slices were then cut in
120 half separating the buccal and lingual surfaces. Each non-carious slice had a flat tipped 300

121 μm diameter needle (Septodont, France) clamped perpendicularly on the external enamel
122 surface before coating the surface with commercially available nail varnish. The buccal/
123 lingual enamel was selected as these areas provided multiple samples containing the best
124 distinction between radial and decussating enamel to detect the schmelzmuster level
125 demineralisation *e.g.* occlusal enamel contains rods that often change direction, which could
126 have made the distinction more difficult to observe. Removal of the needle left an $\sim 300 \mu\text{m}$
127 diameter circular non-varnished area on the slice (Figure 1). Twenty-five of these $500 \mu\text{m}$
128 thick-varnished slices were prepared from ten non-cariou teeth; four slices (one per tooth)
129 for the time-lapse digital microradiography, three slices (one per tooth) for the time-lapse
130 PTLM and eighteen slices (six per tooth) for the pH micro-CT study. In addition, three 500
131 μm thick slices from three carious teeth (one per tooth), containing non-cavitated smooth
132 surface carious lesions were prepared. All samples were stored in phosphate-buffered saline
133 (PBS) prior to use.

134 **2.2 Sample demineralisation as a function of time**

135 Tooth slices for *in situ* digital microradiograph imaging were placed in a radiolucent
136 Kapton® sheet (DuPont™, USA) holder (made with 5×4.5 cm pieces) (see supplementary
137 material, Figure A1). Three gutta-percha points (size 40) were glued with cyanoacrylate
138 between the Kapton® sheets (one per edge) to act as spacers to provide a volume for an acid
139 chamber and seal the margins. Lactic acid (10 % v/v, 0.5 mL, pH 2.2) was injected into the
140 opening of the Kapton® holder to induce enamel demineralisation and simulate an
141 accelerated caries process [23]. Finally, the opening of the sample holder was sealed using
142 molten dental ribbon wax to prevent the evaporation of the acid. Samples were then time-
143 lapse imaged into digitally microradiograph using a SkyScan 1172 micro-CT scanner
144 (Bruker, Belgium) at 60 kV and $167 \mu\text{A}$ in the presence of a 0.5mm aluminium filter at
145 ambient temperature (22°C), using 12 times frame averaging (for noise reduction). The

146 sample stage was positioned to provide images containing inter-pixel distances of $3.02\ \mu\text{m}$ (~
147 $6 \times 4\ \text{mm}$ field of view) in all frames as calculated by the instruments internal pixel size
148 calibration. This value was found to have a sampling error below 2% following imaging
149 stainless steel ball bearings of known diameters ($0.3\ \text{mm}$, $0.5 \pm 0.006\ \text{mm}$ $0.9 \pm 0.002\ \text{mm}$
150 and $1.5 \pm 0.002\ \text{mm}$) (Bearing Warehouse Ltd, UK). Time-lapse sequence were captured
151 using multiple frames captured at intervals of 1 h (in total 86 frames). This was time-lapse
152 sequence digital microradiography not time-lapse sequence micro-CT as there was no
153 rotation between frames. Greyscale (mineral content) profiles over time were generated by
154 sampling the digital microradiographs (linear scan) from the enamel free surface edge
155 through the enamel and dentine using a similar methodology to Deblem et al [24]. The
156 distance from the enamel surface to the point where the readings reached a plateau (*i.e.* intact
157 enamel), were used to calculate the demineralisation front advance. The plateau values were
158 identified by fitting a line through the intact enamel part of the scan lines and selecting the
159 first point of the scan line that intercepted the intact enamel fitted line. Similarly, the width of
160 the demineralised lesion was measured by linearly scanning across the subsurface enamel
161 immediately beneath the acid exposure area and measuring the distance between two plateaus
162 (*i.e.* apparently intact enamel). Greyscale values within the demineralised areas were
163 collected using ImageJ's freehand selection tool by selecting the 85h lesion on each
164 individual digital microradiograph to identify changes in the greyscale with time.

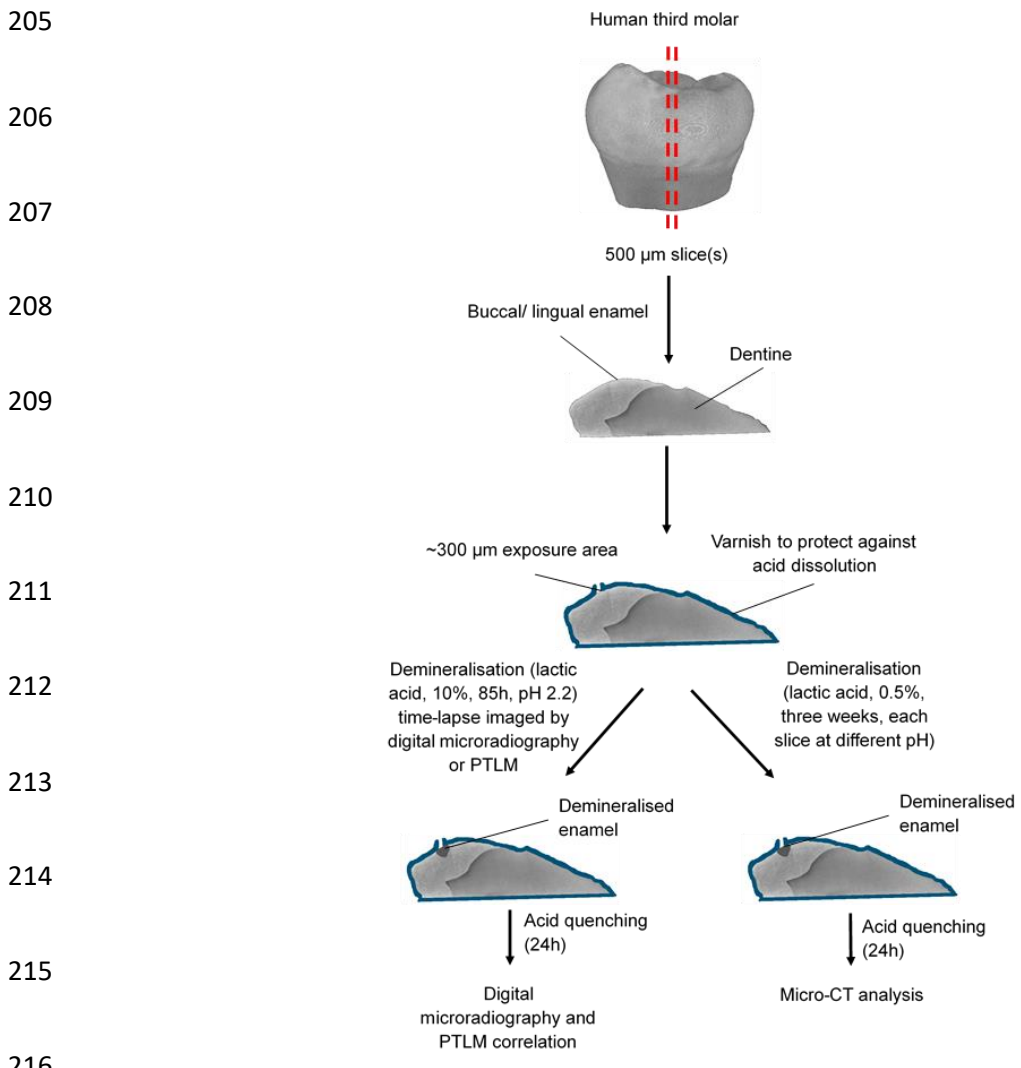
165 Tooth slices for light microscopy *in situ* imaging were glued with cyanoacrylate to the
166 base of a petri dish ($35 \times 10\text{mm}$), to which 5ml of lactic acid (10 % v/v, pH 2.2) was added
167 before securing the petri dish lid with ParafilmTM (to prevent acid evaporation). Samples were
168 then time-lapse imaged (every 100 seconds) using a polarised light microscope (Zeiss
169 Primotech D/A POL, UK) with a 5x objective. The digital microradiography and PTLM
170 experiments were repeated to $N = 4$ and $N = 3$ respectively. The pH was checked after each

171 experiment and remained unchanged. At the end of the *in situ* experiments, samples were
172 placed in PBS (pH 7.4) for 24 h to ensure quenching of the demineralisation process and then
173 imaged using digital microradiography and PTLM for comparison.

174 **2.3 Enamel demineralisation as a function of pH**

175 Varnished tooth slices were incubated at 37°C in lactic acid (0.5% v/v, 0.5mL) (to
176 simulate the carious process [23]) for three weeks at pH 3.6, 4.0, 4.4, 4.8 and 5.2 whilst
177 deionised water (DW) was used as a control with pH 7.0 (one tooth provided six slices, one
178 slice per pH, repeated to N = 3). Solutions were changed every two days to ensure the acidic
179 environment was maintained. The samples were then imaged using the micro-CT method
180 described previously (3.02 µm voxels) except the rotation step was 0.45° with four averaging
181 frames to enable reconstructions as the samples were measured *ex situ* and therefore were
182 micro-CT reconstructions and not digital microradiographs. At the demineralised locations,
183 each sample stayed entirely within the field of view (~ 6 x 4 mm) at each rotation step (no
184 partial volumes). The micro-CT data was reconstructed into Z-stacks using SkyScan's
185 NRecon software (using optimised settings determined qualitatively: smoothing 10, ring
186 artefacts reduction 16, beam hardening 20%, adjusting output maximum 0.2, auto adjusted
187 output minimum) and rendered using ImageJ's Volume Viewer plug-in [25]. The
188 reconstructions were stored in TIFF format and analysed to determine the lesions' depth,
189 width and minimum grey scale values as described earlier. The mineral content of the
190 reconstructions was calibrated to mineral density using six in house made HAp phantom
191 discs. The six HAp discs had densities of 2.76, 2.64, 1.79, 1.67, 1.53 and 0.93 g/cm³ and were
192 made by pressing HAp powder (Sigma Aldrich, UK) in a mould in an engineering vice. The
193 differences in mineral density were achieved by sintering at 800°C, 900°C, 1000°C, 1100°C
194 and 1250°C for 2h (the lowest mineral density phantom was not sintered) to produce the
195 phantom discs 7.4-9.5 mm in diameter and 1-2 mm in thickness. These discs were then glued

216 together using cyanoacrylate and polished to approximately half their original diameters in
 217 order to entirely fit in the micro-CT field of view (see supplementary material, Figure A2).
 218 Greyscale measurements were taken from the reconstructed Z-stack data and measuring the
 219 background greyscale provided a 0.00 g/cm^3 data point. Volumes of the discs were
 220 determined by reconstructing the Z-stacks, thresholding the images, then multiplying the total
 221 number of white pixels by the voxel/ micron ratio. N.B. this mineral density calibration set
 222 was used to quantify mineral density for micro-CT experiments (pH variation) only and not
 223 the digital microradiography experiments (time-lapse sequences) as the calibration discs were
 224 not the same dimensions as the samples.



217 **Figure 1:** Schematic diagram depicting processes for sample preparation.

218 **2.4 Data analysis**

219 Data were plotted in Sigma Plot version 13.0 (Systat Software Inc, San Jose, California,
220 USA) and statistical analysis used the Shapiro-Wilk's test for normality and one-way
221 ANOVA with Dunn's test for multiple comparisons.

222 **3. Results**

223 Micro-CT images generated from the *in situ* experiments were compiled into temporal
224 stacks to visualise enamel demineralisation progression with time (Figure 2). Enamel
225 demineralisation occurred almost instantly but did not progress linearly. Certain enamel
226 regions appeared to be more acid-resistant than others, most notably at the surface. In
227 addition, the demineralisation advance front was not always smooth or rounded, suggesting
228 that preferential demineralisation was dependent on the structural anisotropy of enamel.

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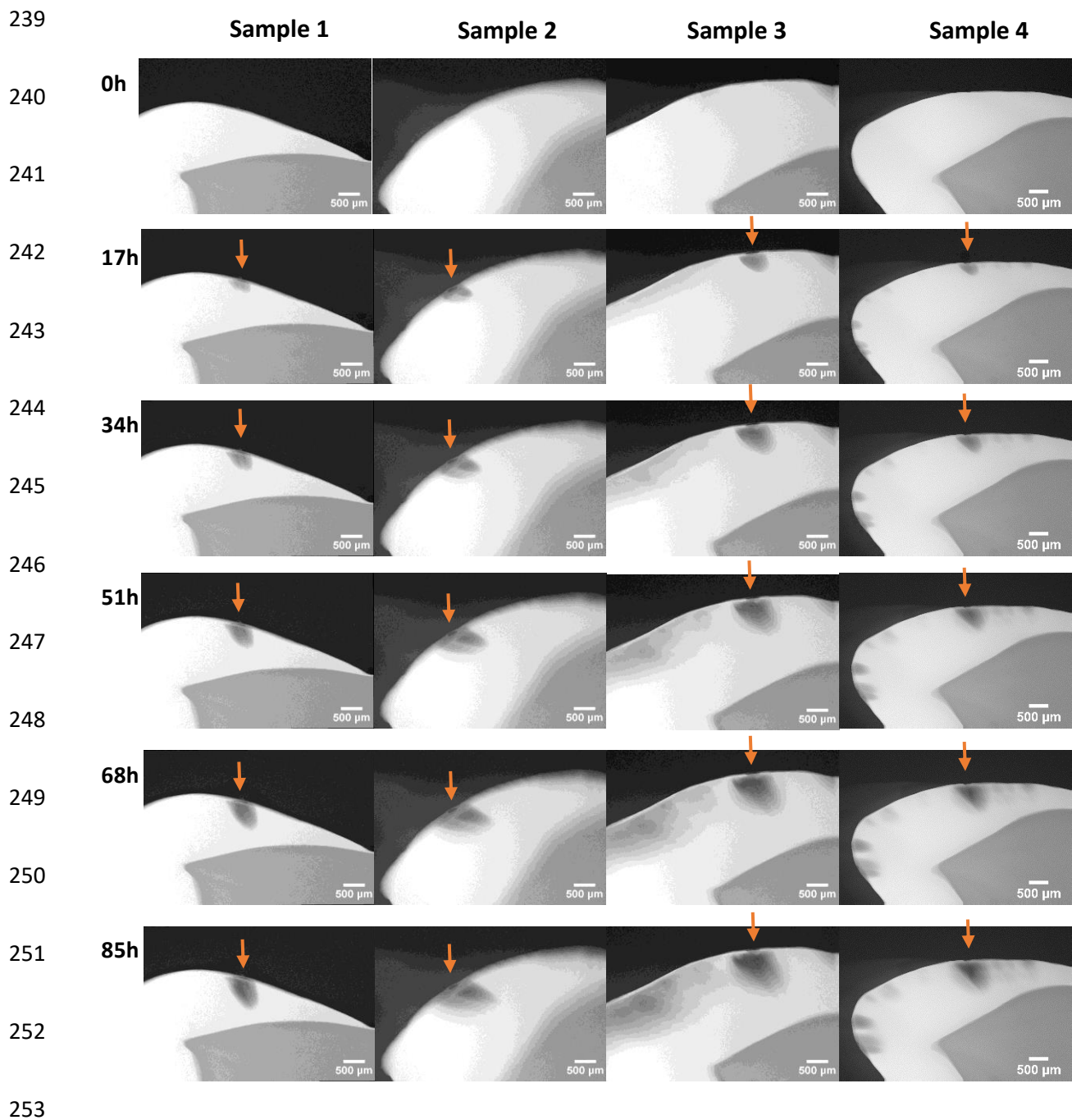
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254 **Figure 2:** Digital microradiography images from time-lapse sequences showing the evolving
 255 demineralisation caused by lactic acid (10% v/v, pH 2.2) in 17h steps. N.B. orange arrows
 256 denote intended demineralisation locations, other areas of demineralisation were due to
 257 incomplete varnish protection. Each sample was prepared from a separate tooth.

258 The demineralisation front kinetics were investigated by monitoring greyscale data
 259 profile lines at 1h intervals (85 hours in total) for the four samples running from the enamel

260 free surface to the dentine (demineralised enamel depth) and across the immediate subsurface
261 enamel (demineralised enamel width), on each time frame (see supplementary material,
262 Figure A3). This enabled calculation of the speed the demineralisation front depth advanced
263 (figure 3A). The data was found to fit with a power-law function ($y = ax^b$, $a = 77.93$, $b =$
264 0.59 , $R^2 = 0.99$). The maximum depth of enamel demineralisation after 85h of exposure was
265 $1042 \pm 198 \mu\text{m}$ at pH 2.2, 0.5 ml and at ambient (22°C) temperature. Measuring the distance
266 across the immediate subsurface enamel on every frame enabled calculation of the speed of
267 demineralisation front width advance (Figure 3B) which also followed a power-law function
268 ($y = ax^b$, $a = 262.05$, $b = 0.34$, $R^2 = 0.97$). Interestingly the b constants in these equations
269 showed the advance in depth of the demineralisation front was faster than the advance in
270 width, indicating a preferential path of demineralisation. In addition, the minimum greyscale
271 (mineral content) in the demineralised enamel areas (Figure 3C) followed an exponential
272 decay function ($y = a * e^{b/(x+c)}$, $a = 5602.50$, $b = 55.17$, $c = 47.04$, $R^2 = 0.99$) with 42%
273 remaining mineral content in the most severely demineralised areas after 85h acid exposure.
274 However, the total rate of greyscale loss from the enamel (figure 3D) was linear ($y = y_0 + ax$,
275 $y_0 = -0.60$, $a = 0.34$, $R^2 = 0.99$) which indicated a continuous but anisotropic
276 demineralisation.

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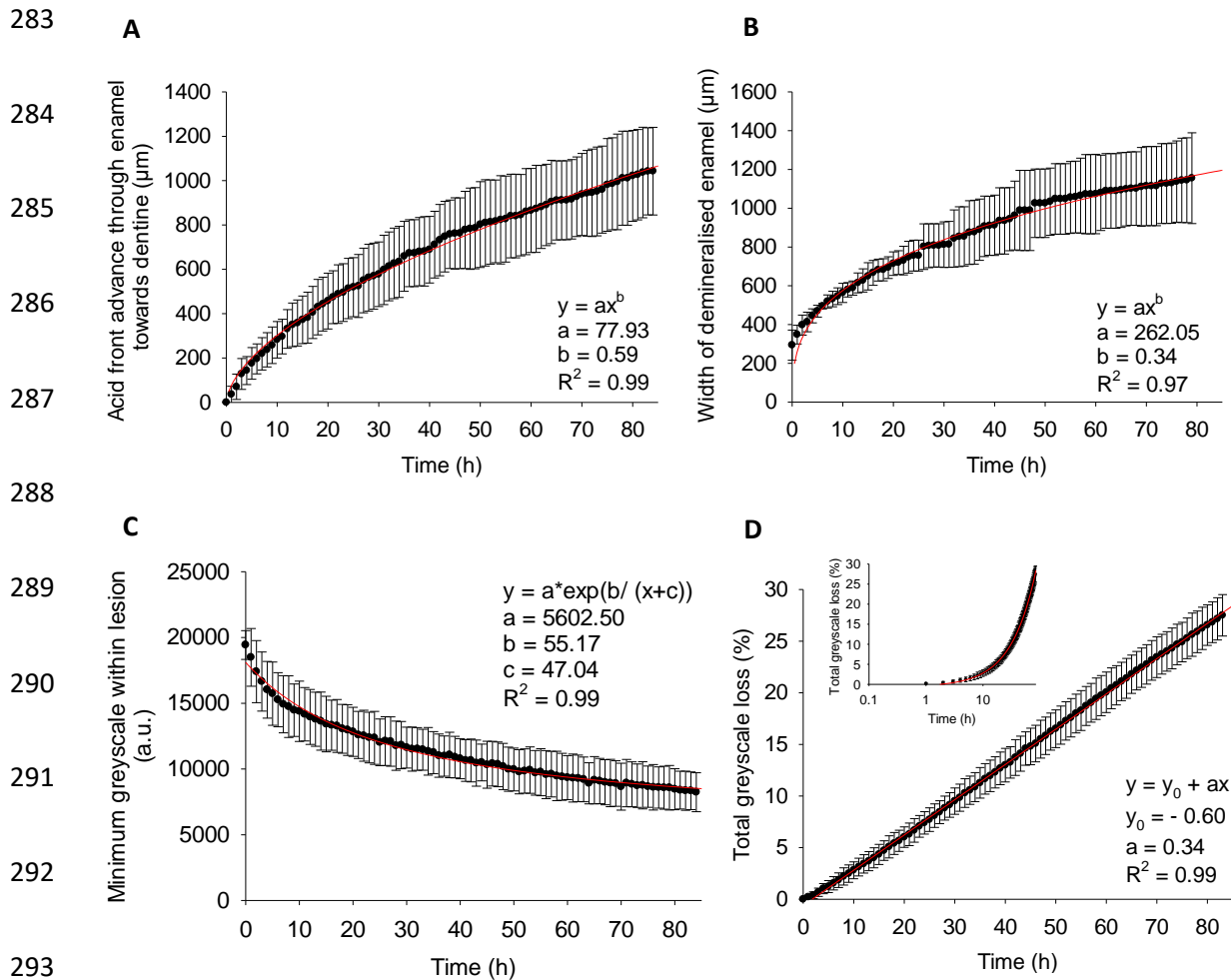
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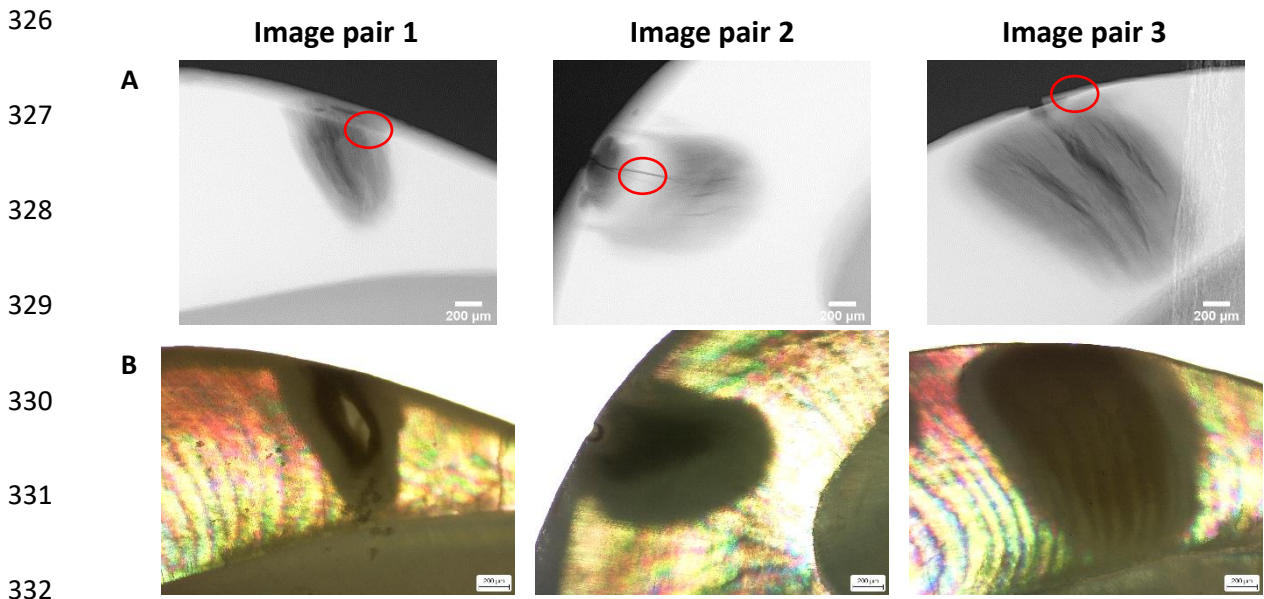
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283 **Figure 3:** Using lactic acid (10% v/v, pH 2.2), the progression of the demineralisation front
 284 depth (A) and width (B) followed power-law processes. The minimum mineral density
 285 remaining within lesions demonstrated an exponential decay model (C), whilst the rate of
 286 total mineral loss (D) was linear showing that a type of mineral environment within prismatic
 287 enamel structure was preferentially demineralised at a constant rate (data presented show
 288 mean \pm standard deviation). Note the lag time during the first few hours (D) and hence
 289 negative y-intercept, showed an initial inhibition of mineral release, the log plot inset
 290 highlights this.

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 294 Light microscopy time-lapse sequences showed that as the demineralisation advanced,
 295 the front appeared to follow HSBs ($\sim 100 \mu\text{m}$ wide) preferentially and determine the shape of
 296 the lesions (Figure 4).



333 **Figure 5:** *Ex situ* comparison of induced enamel demineralisation detection *via* digital
 334 microradiography (A) and polarised transmitted light microscopy (B) showing that the same
 335 sample demineralisation areas were larger in light images than X-ray images (more sensitive
 336 to light detection) *i.e.* in image pairs 1 and 3, the demineralised areas reached the DEJ
 337 whereas in the digital microradiographs they had not. Surface zones are highlighted with a
 338 red circle in the digital microradiographs.

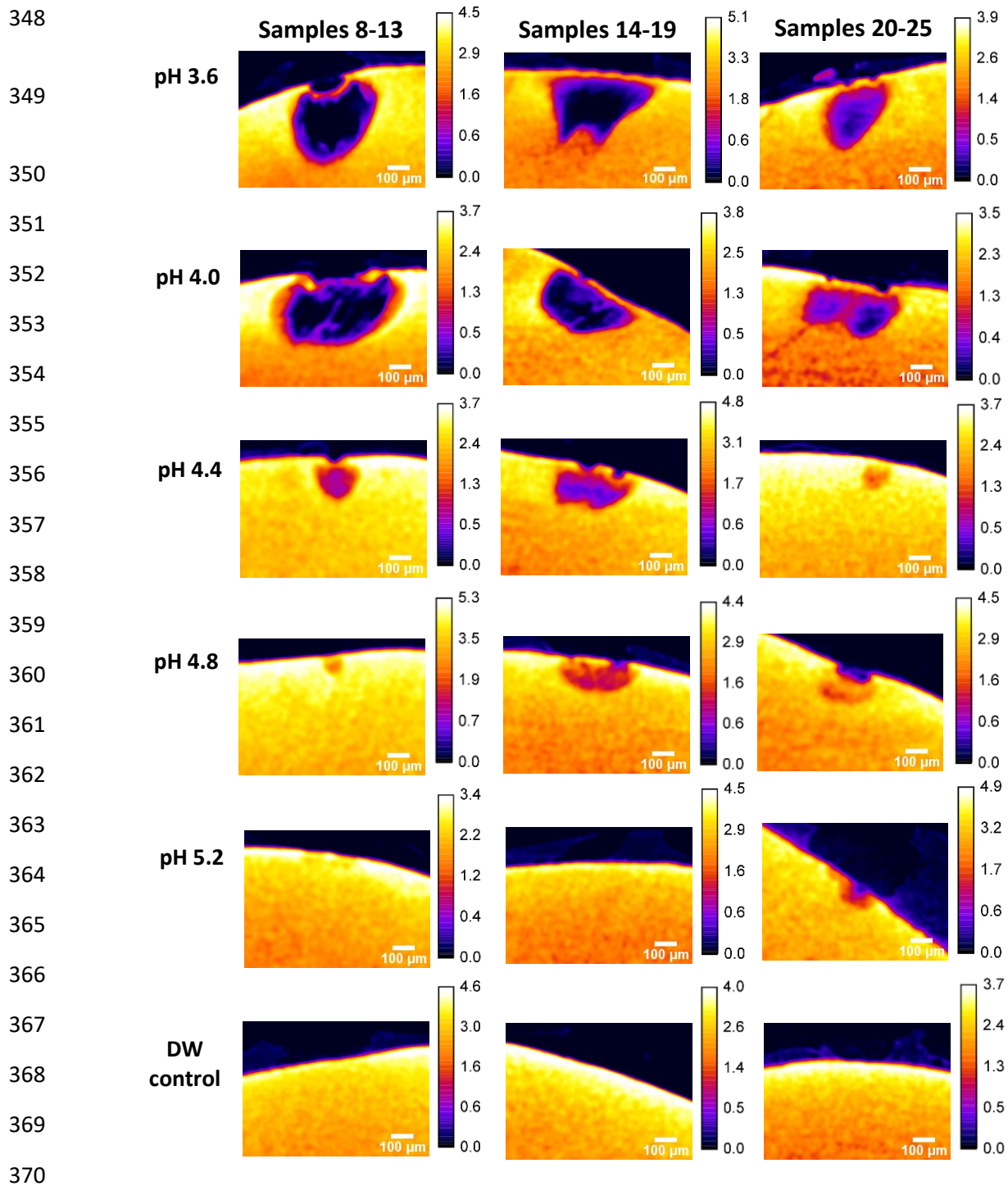
339 Further analysis performed on natural white spot lesions confirmed this, as lesions
 340 were clearly visible using PTLM but not detectable with digital microradiography imaging
 341 (See supplementary material, Figure A4). The effect of pH on enamel (Figure 6) resulted in
 342 larger and more radiolucent lesions with lower pH values and a less radiolucent zone (~ 30
 343 µm thick) remained visible at the surface of most lesions even at the lowest pH [26].

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371 **Figure 6:** *Ex situ* micro-CT 3D reconstructions, orientated perpendicular to slices in ImageJ
 372 volume viewer (and images cropped), of demineralised enamel generated with lactic acid
 373 (0.5% v/v) at a range of pH values after 3 weeks incubation with mineral density calibration
 374 bars inset (g/cm^3). N.B. Edge effect beam hardening at some sample peripheries generated

375 artificially high mineral density values *i.e.* values above $\sim 3.2 \text{ g/cm}^3$. Each column are six
376 samples prepared from the same tooth.

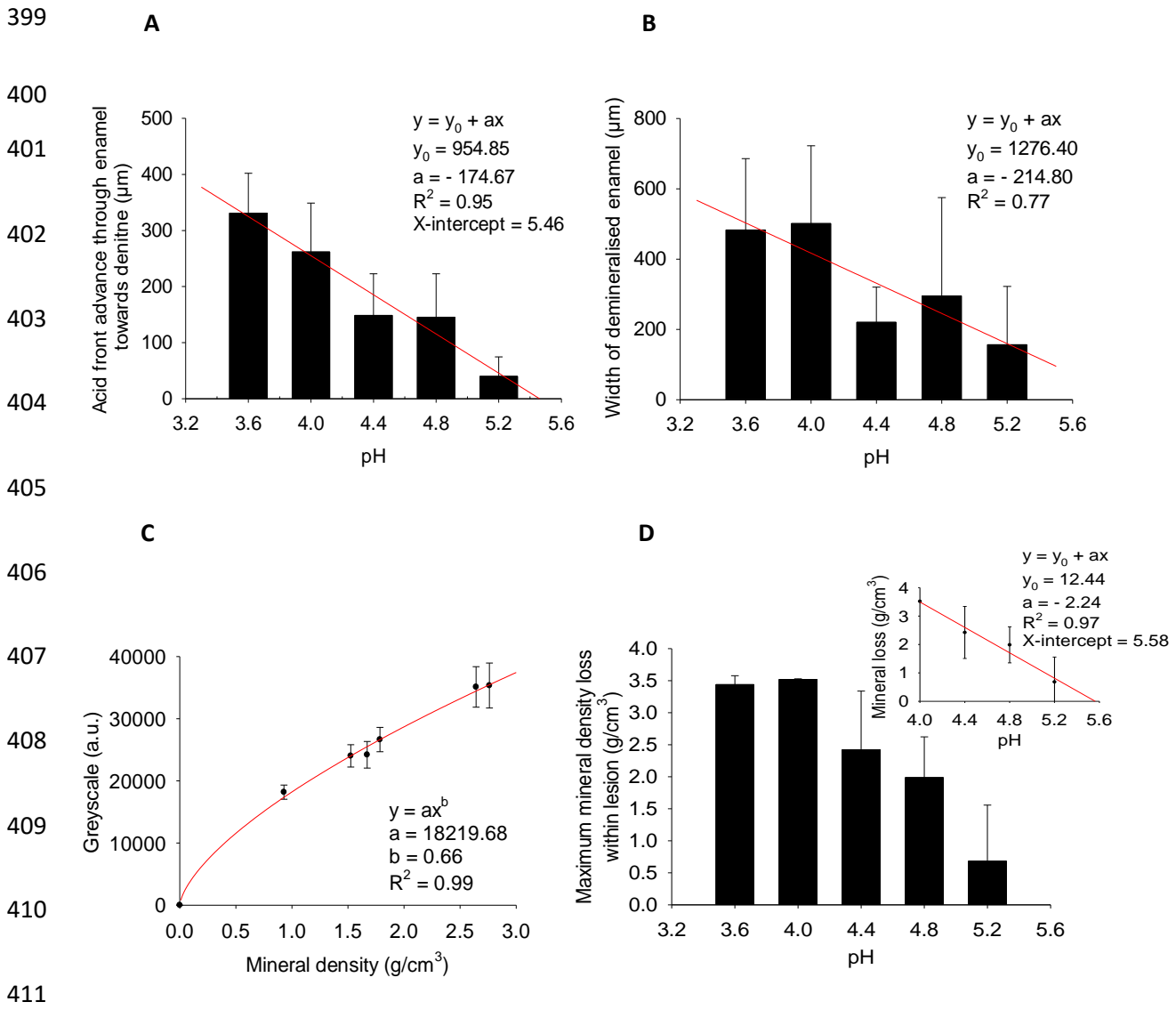
377 Demineralised enamel depth as a function of pH increased as the environment became
378 more acidic linearly ($y = y_0 + ax$, $y_0 = 954.85$, $a = -174.67$, $R^2 = 0.95$) (Figure 7A) and had an
379 x-axis intercept near pH 5.5. The same trend was observed in the demineralised enamel width
380 but the linear function fitted less well ($y = y_0 + ax$, $y_0 = 1276.40$, $a = -214.80$, $R^2 = 0.77$)
381 (Figure 7B) and there was not an x-axis intercept near pH 5.5. This was not surprising, as
382 demineralisation progression laterally across prismatic enamel (permeation across rods, inter-
383 rods and organic sheafs) would be expected to be different to demineralisation progression
384 parallel along prismatic enamel (continuous permeation along rods or inter-rods). In addition,
385 the reason for the non-x-axis intercept at pH 5.5 for width was due to the acid exposure area
386 (hole in the varnish). This was because as soon as a pH initiated enamel demineralisation the
387 entire exposed surface area (width) would have started to become demineralised. The
388 phantom calibration curve was found to have a good fit with the power-law function to link
389 greyscale and mineral density ($y = ax^b$, $a = 18219.68$, $b = 0.66$, $R^2 = 0.996$) (Figure 7C).
390 Interestingly there was a linear relationship between mineral density and pH between pH 5.2-
391 4.0 with an x-axis intercept at \sim pH 5.5 ($y = y_0 + ax$, $y_0 = 12.44$, $a = -2.24$, $R^2 = 0.97$) (figure
392 7D) whereby there was no mineral density at the centre of the demineralised enamel at pH
393 4.0 and below. There appeared to be a correlation between complete mineral loss at the centre
394 of the lesions with a substantial increase in demineralised enamel area.

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412 **Figure 7:** Dose-dependent response of lactic acid pH (0.5% v/v) with demineralised lesion
 413 depth (A), width (B) and maximum mineral density release (D). The plot D inset highlights a
 414 linear mineral release with pH acidity between ~ pH 5.5 (~ critical HAp pH/ x-axis intercept)
 415 and pH 4.0 (complete mineral loss). HAp phantom discs mineral density greyscale calibration
 416 (C). Data presented show mean \pm standard deviation.

417 **4. Discussion**

418 Micro-CT equipment has been widely used to assess bone and dental tissue mineral
 419 density [27] and has been utilised by Davis et al to observe 3D *in situ* tooth demineralisation

420 [28]. The voxel size used by Davis et al [28] was 30 μm with a capture time of 2.6h, whereas
421 in this study, micro-CT was used to capture *in situ* digital microradiographs (2D) at a pixel
422 size of 3.02 μm with an \sim 1 minute capture time at each time point. This provided image
423 resolution 10 times greater than Davis et al with more frequent time points. This allowed the
424 accurate detection of the schmelzmuster mineral loss dynamics and enhanced correlation with
425 PTLM. The data showed that lactic acid almost instantly induced disruption of the enamel
426 surface and a continuous acid exposure resulted in preferential demineralisation of the sub-
427 surface enamel, as previously observed in dental caries [29]. This advanced the work of
428 Klinger & Wiedemann [20] and Davis et al [28], which generated enamel erosion data
429 (complete surface mineral loss causing enamel thinning) possibly due to the use of a larger
430 enamel exposure area to the acidic environment.

431 The surface zone feature of dental caries has been traditionally described [21]. It is
432 thought to form due to a significant amount of fluorine (from toothpaste and mouthwashes)
433 substituting hydroxyl groups in HAp crystallites producing fluorhydroxyapatite (FHAp) or
434 fluorapatite (FAp) which occurs preferentially at the enamel free surface [30]. FAp has a
435 lower average acid solubility than HAp, *i.e.* it has a critical pH of 4.5 compared with 5.5 for
436 HAp [31] and consequently, regions of enamel with less FAp are more susceptible to acid
437 dissolution. In addition to being chemically more resistant, the surface of enamel can be
438 aprismatic in structure [32], and our data shows acid solubility of HAp is affected by
439 crystallite orientation (Figure 4), therefore crystallite spatial arrangement can make the
440 enamel surface physically (and naturally) more resistant to acids [17]. If the surface zone
441 were only observed above the critical pH of FAp this would indicate only chemical
442 resistance. However, a surface zone was observed also in lesions below the critical pH of
443 FAp, which suggested that its existence did not depend exclusively on composition
444 (FAp/FHAp ratios). Furthermore, the surface zone was not exclusively due to the existence of

445 aprismatic enamel as a surface zone was generated in enamel where the surface was partially
446 ground. Therefore, the surface zone phenomena is at least partially influenced by another
447 factor.

448 Sample 1 featured a lesion with a rounded demineralisation front at 85h whilst
449 samples 2-4 had non-rounded fronts. This suggested that demineralisation progressed more
450 isotropically in sample 1 and less so in samples 2-4. The reason for this variation between
451 samples can again be explained by variations in HAp crystallite architecture (crystallite
452 orientations that the acid meets) between samples. In the light microscopy experiments, these
453 preferential demineralised front advances coincided with the HSBs. The HSBs are an optical
454 effect caused by a change in the orientation of adjacent groups of enamel rods (and
455 consequently crystallite orientation) [13]. The HSBs tend to be detectable in the inner two
456 thirds of enamel, which may explain why the non-rounded shaped demineralisation front was
457 observed several days into the experiments (once the acid had reached the HSBs). This is not
458 the only place where preferential demineralisation takes place; it is well established that
459 caries also spreads laterally when reaching the amelo-dentinal junction [33], however, the
460 preferential demineralisation along the HSBs does not appear to have been reported before.

461 The demineralisation front advancement followed a power-law function (regardless
462 of the shape of the lesion), this function had been hypothesised in a mathematical model of
463 the progression of dental caries that our data and observations supported [34]. In addition, the
464 minimum mineral density remaining in the lesions was found to follow an exponential decay
465 model, which suggested preferential loss of a particular HAp orientation [8], [9] *i.e.*
466 preferential enamel rod demineralisation leaving an intact inter-rod matrix behind as the acid
467 front continued to advance. However, the total mineral loss was found to be linear over time
468 hence the rate of lesion growth slowing and exponential decay model for minimum mineral
469 density remaining were not due to diffusion inhibition of the mineral release over time but to

470 an increase in the acid front periphery area *i.e.* the number of rods in contact with the acid. In
471 addition, the 21% higher demineralisation rate in depth over width again suggested that acid
472 preferentially progressed along the enamel rods rather than across them. Also, whilst the total
473 mineral release over time demonstrated a linear relationship it was noted that there appeared
474 to be an initial lag period which could have been related to the generation of the surface zone
475 *i.e.* the presence of FHAp/ FAp and aprismatic enamel. Alternatively, this could have been
476 the observation of type 2 demineralisation transitioning to type 1 as seen in enamel caries
477 [17].

478 Dental caries results in porosity detectable by micro-CT [28] as well as light
479 microscopy [21]. However, our observations indicated that light refraction identified earlier
480 changes in porosity than radiolucency changes detected by standard X-rays or even more
481 sensitive micro-CT equipment. It might therefore be feasible to develop sensitive light-based
482 caries detection systems rather than radiology based techniques. One issue with light-based
483 diagnostics is the need for transmission through the tooth. While this was possible for 500 μm
484 slices, it is more difficult to achieve when imaging whole teeth. Nevertheless, new non-
485 ionising light based diagnostic tools [35] such as optical coherence tomography (OCT) are
486 potentially clinically promising for early stage surface softening detection [36] but the
487 penetration limitation does not allow high-resolution bulk enamel demineralisation imaging.

488 Published micro-CT data [37] shows that healthy enamel has a typical mineral density
489 range between 2.65 – 2.89 g/cm^3 and can be as high as 3.2 g/cm^3 [38]. The top mineral
490 density (2.76 g/cm^3) of the phantom within this work was therefore within the required range
491 for healthy enamel mineral density. However, beam hardening at the edge of some tooth
492 samples induced an artificially high mineral density at some enamel surfaces resulting in all
493 the calibration bars being extrapolated beyond 3.2 g/cm^3 in figure 6 (note that images were
494 still not saturated in those areas). This did not mean the surface zones observed were artefacts

495 as the surface zones were thinner than the beam hardening areas. In order to overcome the
496 beam hardening issue the control mineral density was calculated below the beam hardening
497 area of the samples and produced a mineral density value of $3.51 \pm 0.46 \text{ g/cm}^3$ which was
498 slightly above the previously published mineral density range but was considered acceptable
499 for trend analysis with pH. Enamel carious lesions in the literature have reportedly reduced
500 HAp mineral densities between $1.48 - 2.03 \text{ g/cm}^3$ (losing between $0.62 - 1.41 \text{ g/cm}^3$)
501 compared with the surface zone retaining mineral density between $2.23 - 2.58 \text{ g/cm}^3$ (losing
502 between $0.31 - 0.66 \text{ g/cm}^3$) [38]. This showed that the loss of mineral density in this work
503 was more severe than occurs in natural carious lesions at every pH except the control.

504 However, the linear release of enamel mineral density with increasing pH acidity
505 indicated a logarithmic relationship between mineral loss and proton concentration (due to
506 the logarithmic nature of pH units). This suggested that enamel HAp crystallite orientation
507 has a logarithmic relationship with acid solubility. The intercept of the linear mineral release
508 trend at $\sim \text{pH } 5.5$ supported the linear model because this is near the critical pH of enamel.
509 Previous literature showed that the rate of mineral release over time at different pH values
510 was linear and that the pH affected the gradient of the mineral release linear equations [39]
511 but our work showed that differences in pH environment also had a linear relationship with
512 mineral release.

513 **5. Conclusions**

514 The present study quantified human enamel demineralisation dynamics using an acid-
515 only caries model as a function of time and pH which revealed the total mineral loss with
516 time was linear, whilst the minimum mineral density remaining within demineralised areas
517 demonstrated exponential decay. This showed that a type of mineral environment within
518 prismatic enamel structure *i.e.* rod or inter-rod was preferentially demineralised at a constant

519 rate. Comparison of X-rays with PTLM elucidated the importance of schmelzmuster level
520 HAp crystallite orientation (HSBs) in enamel demineralisation progression and showed light
521 detection is more sensitive to enamel demineralisation than X-rays. The linear rate of mineral
522 loss within the demineralised areas as a function of pH elucidates the relationship between
523 HAp orientation and acid solubility. *In vivo* it is likely that in a severely demineralised
524 enamel, the remaining prismatic scaffold might be susceptible to destruction by masticatory
525 forces, but nonetheless opens the possibility for developing effective therapeutic
526 remineralisation regimes at early stages of demineralisation.

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