Enamel demineralization and remineralization under plaque fluid-like conditions – a QLF study

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Declaration of Interests

The authors declare that there is no conflict of interest.

Abstract

The present study investigated de- and remineralization in enamel lesions under plaque fluid (PF)-like conditions using quantitative light-induced fluorescence (QLF). Pre-formed lesions were exposed to partially saturated lactic acid solutions, varying in pH and fluoride concentration ([F]) based on a 5×3 factorial study design (0/0.1/0.5/1.5/4ppm F;pH 4.9/5.2/5.5). Average fluorescence loss (ΔF) was monitored for 11 days. Subsequently, lesions were demineralized in a partially saturated acetic acid solution for two 24h periods. Data were analyzed using repeated measures analysis of covariance. Lesions exposed to PF at 4ppm F and pH 5.5 showed not only the most remineralization ($\Delta\Delta F$=28.2±14.0%) for all groups after 11d, but also the most demineralization ($\Delta\Delta F$=-19.3±13.5%) after subsequent acetic acid exposure. Increased [F] resulted in more remineralization regardless of pH. Higher pH values resulted in more remineralization. No remineralization was observed in lesions exposed to F-free solutions, regardless of pH. Remineralization was noticeable under the following conditions: pH 4.9 – [F]=4ppm, pH 5.2 – [F]≥1.5ppm, pH 5.5 – [F]≥0.5ppm. Overall, [F] had a stronger effect on remineralization than pH. Subsequent demineralization showed that little protection was offered by PF-like solutions, and further demineralization compared to baseline was observed on lesions not remineralized initially. [F] had a stronger effect on net mineral change than pH. The present study has shown that QLF is a valuable tool in studying lesion de- and remineralization under PF-like conditions, where [F] was shown to be more important than pH.

Introduction

In vitro de- and remineralization of enamel caries lesions in the presence or absence of fluoride have been studied using a broad range of approaches, which were subject to comprehensive reviews in the past [Featherstone, 1996; ten Cate, 1990]. In general, these studies can be divided into two groups – studies using pH cycling models with samples being subjected to alternate treatments with de- and remineralization solutions [ten Cate and Duijsters, 1982], which have been developed further to study the comparative effectiveness of (fluoride) treatments in preventing demineralization and/or enhancing remineralization of caries lesions [White, 1987, 1988] or, studies using constant composition conditions to study the inherent de- or remineralization effectiveness of a solution [ten Cate and Arends, 1977]. Typically, remineralizing solutions either mimicking human saliva in its chemical composition or resembling hydroxyapatite stoichiometry and demineralizing solutions resembling high cariogenic challenges are employed in these studies, and especially in pH cycling protocols. However, conditions at the sites of caries occurrence; i.e. at the plaque enamel interface, are somewhat different. Studies on the composition of plaque-fluid (PF) (i.e. the aqueous, extracellular phase of plaque) have found marked differences between the chemical composition of PF and that of human saliva [Carey et al., 1986; Gao et al., 2001]. In particular, pH, Ca, F and Pi concentrations as well as the Ca/Pi ratio in PF were shown to be dissimilar to human saliva.

Little research has been conducted investigating the effect of PF-like systems on lesion de- and remineralization, which, essentially, are de- and remineralization systems close to saturation with respect to enamel and hydroxyapatite. Previous, relevant studies [Lynch et al., 2006; Yamazaki et al., 2007] have shown that these systems are very sensitive to small changes in pH and especially fluoride in terms of the net outcome – further de- or remineralization of the lesion.

Despite being a relatively new interrogation technique, quantitative light-induced fluorescence (QLF) has been employed in caries research in a broad range of applications: pH cycling studies [Hafstrombjorkman et al., 1992], studies on very early demineralization [Ando et al., 1997], in monitoring in vivo remineralization of white spot lesions in orthodontics patients [Al-Khateeb et al., 1998] and in studies on lesion activity [Ando et al., 2006] to name only a few. Comparative

studies in relation to the "gold standard" technique transverse microradiography (TMR) have been conducted, and good correlations were shown by various investigators [Ando et al., 2001; Fujikawa et al., 2008; Hafstrombjorkman et al., 1992]. One of the major advantages of QLF over TMR is the ability to nondestructively and longitudinally monitor progression and reversal of caries lesions without having to compromise on the "window width" of the lesion, which has been shown to be a very important factor in, at least, the demineralization of the dental hard tissues [Ruben et al., 1999]. Repeated TMR analysis on the same specimen is only possible using "single section" [Tanaka et al., 1993] or "sandwich" [Mellberg et al., 1986] models, both compromising on the optimum lesion "window width".

Therefore, the aim of the present study was to build on the findings of a previous, relevant study [Lynch et al., 2006], by longitudinally monitoring of de- and remineralization of artificial carious lesions under PF-like conditions in vitro using QLF.

Materials and Methods

Enamel Specimen Preparation

Enamel blocks, approx. 5×5mm in size, were prepared from sound, bovine incisors and embedded in epoxy resin ('EpoxyCure'; Buehler, Coventry, UK). The labial surfaces were abraded to a depth of approx. 0.5mm to expose bulk enamel, and specimens were then polished using P800 silicon carbide abrasive discs on a water-cooled grinder-polisher ('AutoMet'; Buehler, Coventry, UK). Two pieces of clear, solvent resistant, self-adhesive tape ('853'; 3M, Manchester, UK) were placed across two opposite sides of the specimens, thus exposing an experimental window of approx. 5×2mm in size. Prepared specimens were stored at 100% relative humidity until use.

Lesion Preparation

Subsurface caries lesions were created in specimens using a modified acid gel method based on that used by Laboratory D as described elsewhere [ten Cate et al., 1996]. Specimens were placed in containers and covered with approx. 2.5cm of an 8% methyl cellulose ('M0387';

Sigma-Aldrich, Dorset, UK) gel which was left to set at 4°C overnight. The gel was then covered with an equal volume of 0.1M lactic acid, pH adjusted to 4.6 with KOH. The containers were sealed and placed in an incubator at 37°C for 12 days. After the demineralization period, specimens were removed from the gels, rinsed using deionized water and stored at 100% relative humidity until further use.

QLF Measurements

All specimens were air-dried for at least 30min at room temperature before QLF measurements were performed using the 'QLF In Vitro' system (Inspektor Research Systems BV, Amsterdam, the Netherlands), which is described elsewhere [Gmur et al., 2006]. As the lesion window was identical in size for all specimens, only average fluorescence loss (ΔF) values were recorded and at a threshold level of 5%, i.e. a minimum of 5% fluorescence loss between sound and demineralized enamel. The distance between the camera and the surface of the enamel block was kept constant throughout the experiment to facilitate repeat measurements. Specimens were randomized into 15 treatment groups (n=9 per group) based on ΔF after lesion creation. Repeat Measurements on specimens were performed after 2, 4, 7, 9 and 11 days of exposure to PF-like solutions, and after 24 and 48 hours of exposure to the partially saturated acetic acid solution. QLF images were superimposed to accurately calculate $\Delta\Delta$F values for each specimen. $\Delta\Delta$F values were calculated using the following equations:

$\Delta\Delta F_{PF} = \Delta F_{PF(11d)} - \Delta F_{Base}$

(describes extent of de- or remineralization of lesions after PF-exposure in relation to lesion baseline)

$\Delta\Delta F_{PSAc} = \Delta F_{PSAc(48h)} - \Delta F_{PF(11d)}$

(describes extent of demineralization of lesions after acetic acid exposure in relation to lesions after PF-exposure)

$\Delta\Delta F_{net} = \Delta\Delta F_{PF} + \Delta\Delta F_{PSAc} = \Delta F_{PSAc(48h)} - \Delta F_{Base}$

(describes extent of de- or remineralization of lesions after PF- and acetic acid exposure in relation to lesion baseline)

Thus, positive $\Delta\Delta$F values indicate remineralization, whereas negative $\Delta\Delta$F values indicate (further) demineralization.

Plaque Fluid (PF)-like Solutions

A total of 15 different solutions, based on a 5×3 factorial study design, and simulating PF in its chemical composition were prepared. All solutions contained 30mM lactic acid, 5.5mM calcium chloride dihydrate, 9.4mM potassium dihydrogen phosphate and 63mM potassium chloride, but varied in fluoride concentration (0/0.1/0.5/1.5/4ppm F as NaF) and pH (4.9/5.2/5.5 – adjusted using KOH). Fluoride to hydrogen ion molar ratios were calculated for all solutions and are shown in table 1. Solutions and groups of specimens treated with these solutions were coded in the present study based on [F] and pH; e.g. "0.5F/pH4.9" would describe a solution containing 0.5ppm F and having a pH of 4.9 and also a group of nine specimens treated with this solution. Specimens were exposed to these solutions at 37°C for 11 days with the solutions being renewed after 2, 4, 7 and 9 days; i.e. after QLF measurements were performed.

Partially Saturated Acetic Acid Solution (PSAc)

A partially saturated acetic acid solution [Lynch and ten Cate, 2006] containing 50mM acetic acid, 2.25mM calcium chloride dihydrate, 1.35mM potassium dihydrogen phosphate and 130mM potassium chloride, pH adjusted to 5.0 with KOH was prepared. After 11 days of exposure to PF-like solutions, all specimens were exposed to the acetic acid solution at 37°C for two consecutive 24 h periods on a group by group basis.

Calculation of Saturation with Respect to Calcium Phosphate and Fluoride Phases

The solutions' respective degrees of saturation with respect to hydroxyapatite (DS_{HA}), octacalcium phosphate (DS_{OCP}), brushite (DS_{BR}), fluorapatite (DS_{FA}) and calcium fluoride (DS_{CaF2}) were calculated using a computer program [Shellis, 1988] and are shown in table 1.

Statistical Analysis

Comparisons between different pH values and fluoride concentration combinations were compared using a repeated measures analysis of covariance. The repeated ANCOVA model included ΔF as the response variable and factors for pH, fluoride concentration [F], time and $ΔF_{Base}$ as a covariate. Interaction terms were also included for pH×[F] and pH×[F]×time. These interaction terms provided the comparisons between the 15 different combinations of pH with [F]. The model was broken out into a remineralization phase and a demineralization phase. The

remineralization phase was over days 2 to 11 (with day 0 as a baseline covariate). The demineralization phase was over days 11 to 13 (with day 0 as a baseline covariate). The software SAS v8.2 (SAS Institute Inc., Cary, N.N., USA) was used to analyse the data.

Results

Statistical analysis of QLF data revealed statistically significant interactions between pH×[F] and pH×[F]×time ($p < 0.001$). The results of all QLF measurements and calculated $\Delta\Delta F$ values are shown in figure 1. The average ΔF ($\pm$ SD) for all specimens at lesion baseline was -55.6 $\pm$ 6.2%. Numerically, group "4F/pH5.5" showed not only the highest $\Delta\Delta F_{PF}$ (28.2 $\pm$ 14.0 %) for all groups, but also the highest $\Delta\Delta F_{PSAc}$ (– 19.3 $\pm$ 13.5 %). $\Delta\Delta F_{net}$ values were negative for all fluoride-free groups, those containing 0.1ppm F and group "0.5F/pH4.9". All other groups showed positive $\Delta\Delta F_{net}$ values. For specimens of all groups, no relationship between ΔF_{Base} and ΔF_{PF} was observed ($r = -0.16$). However, there was a strong relationship between $\Delta F_{PF(11d)}$ and $\Delta F_{PSAc(24h)}$ ($r = -0.72$).

Relatively small changes in $\Delta\Delta F$ were observed on all specimens during the first four days of exposure to PF-like solutions. Then, an almost exponential increase in $\Delta\Delta F$ values was noted in 0.5F, 1.5F and 4F groups, before plateauing in the 4F group. The first 24h PSAc exposure led to a sharp decrease in $\Delta\Delta F$ values, and especially for specimens showing increased $\Delta F_{PF(11d)}$ values (i.e. specimens that showed considerable remineralization after 11 days of PF exposure). However, comparatively little difference in $\Delta\Delta F$ between 24 h and 48 h PSAc was observed for all specimens.

The results of the statistical analyses of the PF data are presented in table 2~~3~~. For better visualization of treatment differences, figure 2 shows the overall pH and F effects during PF and combined PF and PSAc exposures on $\Delta\Delta F$. For the different F concentrations and pH ranges tested, F had a stronger effect on $\Delta\Delta F_{PF}$ than pH; a difference in [F] of 1ppm had the same effect as a difference in 0.6 pH units. Linear pH ($r = 1.00$) and [F] ($r = 0.99$) $\Delta\Delta F_{PF}$ relationships were found. Furthermore, strong relationships were found for DS_{FA} and $\Delta\Delta F_{PF}$ ($r = 0.70$) for groups

treated with F-containing solutions, and for DS_{HA} and $\Delta\Delta F_{PF}$ ($r = 0.79$) for groups treated with F-free solutions.

The results of the statistical analyses of the PSAc data are presented in table 34. The statistical analysis of ΔF_{PSAc} data at 13d are equivalent to the statistical analysis of the $\Delta\Delta F_{net}$ data. Again, [F] had a stronger effect than pH (on $\Delta\Delta F_{net}$). A linear pH $\Delta\Delta F_{net}$ relationship ($r = 0.99$) was found, whereas the [F] $\Delta\Delta F_{net}$ relationship followed an exponential pattern ($r = 1.00$).

Variability in ΔF measurements for groups showing remineralization increased with exposure time. As an example for all these groups, ΔF values for each specimen of group "4F/pH5.5" vs. time are presented in figure 3.

Discussion

The design of the present study was based on previous investigations [Lynch et al., 2006; Yamazaki et al., 2007] with the aim to further build on F and pH effects in PF-like solutions on the de- and remineralization of caries lesions by investigating F effects over a smaller range, and pH effects over a broader range, than reported previously, thus increasing the physiological relevance. Furthermore, the acid resistance of the lesions post-PF exposure was investigated. The compositions of the PF-like solutions used in the present study were loosely based on the aforementioned studies and were similar to the chemical composition and pH range reported for plaque fluid (4.97 – 5.45) [Carey et al., 1986; Gao et al., 2001]. To facilitate repeated QLF measurements, "simple" solutions rather than a gel system [Blake-Haskins et al., 1992; Lynch et al., 2006] were used for the PF treatments, thus resembling more smooth surface caries. Baseline lesion severity (ΔF_{Base} was among the lowest reported in the literature), taking into account the solutions' DS values (table 1), as well as lesion mineral distribution (methyl cellulose acid gels yield high-R lesions) were chosen to allow for better treatment discrimination [Lynch et al., 2007; Lynch and ten Cate, 2006]. So, although the physiological relevance of the study would have been enhanced by the presence of a plaque-mimetic, in terms of both modified mineral diffusion and reduction in ionic activity, the chosen study design was optimized for a better

mechanistic understanding of the impact of [F] and pH on de- and remineralization of caries lesions under PF-like conditions over time.

No net demineralization post PF-treatments occurred in any of the experimental groups, which was not surprising, considering $DS_{HA} > 1$ for all PF-like solutions. Some marked differences in DS_{HA}, DS_{OCP} and DS_{BR} between the different solution pH values, as well as the relatively small differences in DS_{FA} between the different [F] compared to DS_{FA} differences between pH values tested, would have suggested a stronger pH than F effect on $\Delta\Delta F_{PF}$; however, the opposite was the case as [F] had a considerably stronger effect than pH in the present study (figure 2). These findings are in agreement with a previous study [Lynch et al., 2006], both highlighting that [F] is a more important driving factor than pH for net remineralization under conditions resembling plaque fluid, and that even small elevations in [F] (0.5 to 1.5 ppm) can compensate for relatively large pH differences (5.5 to 4.9) as seen in the present study. In other words and extrapolating this to the clinical situation, successful remineralization strategies should be predominantly based on delivering effective amounts of fluoride – regardless of the application pH. Furthermore, strategies based on pH buffering agents are less likely to be successful, but may provide an alternative where fluoride cannot be employed. However, further studies employing lower and higher pH values than those employed in the present studies are warranted to fully understand the pH $\times$ [F] interaction.

After 11 days of exposure to PF-like solutions, remineralization was only noted under the following conditions: pH 4.9 – [F] = 4 ppm, pH 5.2 – [F] $\geq$ 1.5 ppm, pH 5.5 – [F] $\geq$ 0.5 ppm (see statistical analysis in table 2). All other groups showed no significant changes from lesion baseline.

The lack of appreciable remineralization in most groups within the first 4 days of PF-exposure and the sudden increase in $\Delta\Delta F_{PF}$ between days 4 and 7, especially for some F groups, was somewhat surprising (figure 13). However, considering the entire 11 day period, almost linear relationships in ΔF vs. PF exposure time were noted in most groups showing net remineralization (figure 1). This is at least partially in agreement with comparable studies [Yamazaki et al., 2007; Yamazaki and Margolis, 2008], both investigating [F] effects on remineralization under acidic,

PF-like conditions and observing almost linear relationships between remineralization time and reduction in lesion volume. The reasons for this discrepancy within the first days of PF exposure and the general variability in lesion response – perceived or not – are not clear. It can only be speculated that either lack of sensitivity of the QLF technique in comparison to TMR at these early stages, or, and perhaps more likely, the general lack of knowledge about the QLF technique in relation to lesion parameters (e.g. surface zone mineralization, "R" value, presence of lamination etc.) and changes thereof may explain this discrepancy. However, for longitudinal studies, it does highlight the value of techniques which complement TMR, which by virtue of its destructive nature currently gives information at a limited number of time-points [Lynch et al., 2007]. To the authors' knowledge, no comparative QLF/TMR studies have been conducted on lesions de- and or remineralized using PF-like systems, but relevant TMR studies [Lynch et al., 2006; Yamazaki et al., 2007; Yamazaki and Margolis, 2008] have shown considerable changes in mineral distribution within the lesions as well as lamination under these conditions. Although QLF has been shown to be fairly robust to changes in surface zone mineralization [Fujikawa et al., 2008], further, comparative studies are required to fully understand the potential of this still relatively new technique.

The comparison of [F] and pH effects on $\Delta\Delta F_{net}$ vs. $\Delta\Delta F_{PF}$ is not straightforward and perhaps impossible (figure 2). Overall pH effects did not appear to differ between the two "effectiveness" measures, but F effects followed a different pattern for $\Delta\Delta F_{net}$ in comparison to $\Delta\Delta F_{PF}$. It must be considered that significant differences existed in ΔF values of lesions prior to PSAc exposure, thus further complicating the interpretation of the data. Lesion baseline characteristics have been shown to have an impact on subsequent de- and remineralization behavior of lesions – lesion baseline demineralization is inversely related to subsequent demineralization; i.e. smaller lesions (by means of lesion severity at baseline) tend to demineralize faster than deeper lesions [Lynch and ten Cate, 2006]. Thus, it was not surprising that lesions showing the highest $\Delta\Delta F_{PF}$ also showed the highest $\Delta\Delta F_{PSAc}$. However, no such relationship was observed between ΔF_{Base} and $\Delta\Delta F_{PF}$, although considerable differences in the responsiveness of lesions to PF treatments, especially those with [F] > 0.1 ppm, were seen (figure 1). Differences in mineral distribution at baseline, and especially differences in the degree of surface zone mineralization, may be an explanation for the varied response to F under these conditions.

Based on the $\Delta\Delta F_{net}$ data, "acquired acid resistance" [Koulourides and Cameron, 1980] of the lesions was observed in relationship to [F] during PF exposure in the present study. Interestingly, a sharp decrease in ΔF after 24 h of PSAc exposure was noted, but very little difference in ΔF was observed between 24 h and 48 h PSAc. Newly deposited mineral may have been rapidly lost during the first 24 h of the post-PF exposure acetic acid challenge, but further demineralization was prevented, suggesting that only a "minor arrest" of the lesions occurred during the PF exposure. Lesion baseline characteristics (the chosen methyl cellulose acid gel methodology yields high-R lesions) may have been a contributing factor as high-R lesions can be considered more difficult to arrest than naturally occurring white spot lesions (which were shown to exhibit low-R values [Lynch et al., 2007]). The comparatively lower degree of surface zone mineralization of high-R lesions complicates the establishment of a surface zone diffusion barrier, and this may at least partially explain the present findings.

Further studies are required employing the (current) gold standard technique TMR and more physiologically relevant study protocols (e.g. use of low-R lesions and lesions with different degrees of mineral loss at baseline, use of experimental setups mimicking plaque better in its physical and chemical properties) to validate the findings of the present study.

In conclusion, the present study has shown that QLF is a valuable tool in studying lesion de- and remineralization under PF-like conditions. Under the conditions of the study, [F] was shown to be more important than pH in lesion remineralization and protection against subsequent demineralization.

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Table 1. Fluoride to hydrogen ion molar ratios and degree of saturation of solutions used in the present study with respect to calcium phosphate and fluoride phases (HA – hydroxyapatite; OCP – octacalcium phosphate; BR – brushite; FA – fluorapatite; CaF2 – calcium fluoride).

Table 2. Statistical analysis of ΔF_{PF} data. Separate results are shown for each pH - [F] combination, at days 2, 4, 7, 9, and 11. Statistically significant differences within pH or F groups are <u>underlined</u> and highlighted in **bold**.

Capital letters represent treatment means comparison within pH group (i.e. comparison within rows), whereas lower case letters represent treatment means comparisons with F group (i.e. comparison within columns).

Table 3. Statistical analysis of ΔF_{PSAc} data. Separate results are shown for each pH - [F] combination, at days 11, 12, and 13. The statistical analysis of ΔF_{PSAc} data at 13d are equivalent to the statistical analysis of the $\Delta\Delta F_{net}$ data. Statistically significant differences within pH or F groups are <u>underlined</u> and highlighted in **bold**.

Capital letters represent treatment means comparison within pH group (i.e. comparison within rows), whereas lower case letters represent treatment means comparisons with F group (i.e. comparison within columns).

Figure 1. Results of QLF measurements for all 15 experimental groups as a function of treatment period and time. Lesions were exposed to plaque fluid-like solutions (PF) for 11d, followed by 2x24h treatments with a partially saturated acetic acid solution (PSAc). NET change vs. lesion baseline after conclusion of experiment was calculated. $\Delta\Delta F$ values were calculated

with respect to ΔF at lesion baseline (-55.6%). Positive values indicate remineralization, whereas negative values are indicative of further demineralization. Error bars were omitted for better clarity.

Figure 2. Overall pH (right axis, unfilled symbols) and F (left axis, filled symbols) effects on $\Delta\Delta F_{net}$ (circle) and $\Delta\Delta F_{PF}$ (square).

Figure 3. ΔF vs. exposure time for all nine specimens treated with the plaque fluid-like solution containing 4ppm F at pH 5.5 (baseline – 0d; treatment with plaque fluid-like solution – 0 to 11d; exposure to partially saturated acetic acid solution – 11 to 13d).

Table 1. Fluoride to hydrogen ion molar ratios and degree of saturation of solutions used in the present study with respect to calcium phosphate and fluoride phases (HA – hydroxyapatite; OCP octacalcium phosphate; BR – brushite; FA – fluorapatite; CaF2 – calcium fluoride).

Solution	F/H molar ratio	DS_{HA}	DS_{OCP}	DS_{BR}	DS_{FA}	DS_{CaF2}
0F/pH4.9	0	1.24	0.49	0.50	0.00	0.00
0.1F/pH4.9	0.42	1.24	0.49	0.50	4.65	0.08
0.5F/pH4.9	2.09	1.24	0.49	0.50	5.55	0.23
1.5F/pH4.9	6.27	1.24	0.49	0.50	6.27	0.48
4F/pH4.9	16.72	1.24	0.49	0.50	6.99	0.91
0F/pH5.2	0	2.09	0.74	0.70	0.00	0.00
0.1F/pH5.2	0.83	2.09	0.74	0.70	7.29	0.08
0.5F/pH5.2	4.17	2.09	0.74	0.70	8.70	0.23
1.5F/pH5.2	12.51	2.09	0.74	0.70	9.83	0.48
4F/pH5.2	33.37	2.09	0.74	0.70	10.96	0.92
0F/pH5.5	0	3.53	1.13	0.97	0.00	0.00
0.1F/pH5.5	1.66	3.53	1.13	0.97	11.37	0.08
0.5F/pH5.5	8.32	3.53	1.13	0.97	13.58	0.23
1.5F/pH5.5	24.97	3.53	1.13	0.97	15.34	0.47
4F/pH5.5	66.57	3.53	1.13	0.97	17.11	0.91
Acetic acid	0	0.46	0.17	0.13	0.00	0.00

Table 2. Statistical analysis of ΔF_{PF} data. Separate results are shown for each pH - [F] combination, at days 2, 4, 7, 9, and 11. Statistically significant differences within pH or F groups are underlined and highlighted in **bold**.

Time [d]	pH	F [ppm]				
		0	0.1	0.5	1.5	4
2	4.9	A - a	A - a	A - a	A - a	A - a
	5.2	A - a	A - a	A - a	A - a	A - a
	5.5	A - a	A - a	A - a	A - a	A - a
4	4.9	A - a	A - a	A - a	A - a	A - a
	5.2	AB - a	A - a	AB - a	AB - a	**B** - a
	5.5	A - a	A - a	A - a	A - a	A - a
7	4.9	A - a	A - a	A - a	AB - a	**B** - a
	5.2	A - a	A - a	A - a	AB - a	**B** - a
	5.5	A - a	A - a	A - a	AB - a	**B** - a
9	4.9	A - a	A - a	A - a	A - a	**B** - a
	5.2	A - a	A - a	A - a	A - a	**B** - ab
	5.5	A - a	A - a	AB - a	**B** - a	**C** - **b**
11	4.9	A - a	A - a	A - a	A - a	**C** - a
	5.2	A - a	A - a	A - a	**BC** - a	**C** - a
	5.5	A - a	AB - a	**BC** - **b**	**C** - a	**D** - a

Capital letters represent treatment means comparison within pH group (i.e. comparison within rows), whereas lower case letters represent treatment means comparisons with F group (i.e. comparison within columns).

Table 3. Statistical analysis of ΔF_{PSAc} data. Separate results are shown for each pH - [F] combination, at days 11, 12, and 13. The statistical analysis of ΔF_{PSAc} data at 13d are equivalent to the statistical analysis of the $\Delta\Delta F_{net}$ data. Statistically significant differences within pH or F groups are underlined and highlighted in **bold**.

Time [d]	pH	F [ppm]				
		0	0.1	0.5	1.5	4
11	4.9	A - a	A - a	A - a	A - a	**B** - a
	5.2	A - a	A - a	A - a	**BC** - ab	**C** - a
	5.5	A - a	A - a	**BC** - **b**	**C** - **b**	**D** - a
12	4.9	A - a	A - a	AB - a	**BC** - a	**C** - a
	5.2	A - a	A - a	AB - ab	**BC** - a	**C** - a
	5.5	A - a	A - a	ABC - **b**	**BC** - a	**C** - a
13	4.9	A - a	A - a	AB - a	**BC** - a	**C** - a
	5.2	A - a	A - a	AB - ab	**BC** - a	**C** - a
	5.5	A - a	A - a	ABC - **b**	**BC** - a	**C** - a

Capital letters represent treatment means comparison within pH group (i.e. comparison within rows), whereas lower case letters represent treatment means comparisons with F group (i.e. comparison within columns).