

# MEASUREMENT OF pH IN ETHANOL, DISTILLED WATER, AND THEIR MIXTURES: ON THE ASSESSMENT OF pH IN ETHANOL-BASED NATURAL HISTORY WET COLLECTIONS AND THE DETRIMENTAL ASPECTS OF DILUTION WITH DISTILLED WATER

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*Abstract.*—The measurement of the pH in alcohol-preserved natural history collections is fraught with considerable theoretical and methodological difficulties. A major problem is the concentration-dependent effect of ethanol. Based on empirical results in connection with data from the literature, this study aims to estimate the shape and magnitude of this effect and suggest an approximate method to correct for it. It surfaces that considerable problems are caused by the use of distilled water for the dilution of preservation fluids.

*Key words.*—collection, conservation, dilution, distilled water, ethanol, mixture, natural history, pH, preservation.

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## INTRODUCTION

Measurement of pH in hydroalcoholic solutions is known to be difficult and riddled with systematic error. The pH scale was developed for describing the acidity of aqueous solutions. Accordingly, pH electrodes are generally customized and calibrated for measurements in aqueous solutions. In the presence of alcohol, however, the conditions in the solution and the behavior of the electrode are altered. Helpful introductions into the various aspects and limitations of pH measurements in hydroalcoholic solutions are provided by Frant (1995), Tindall and Dolan (2002), Sound and Becker (2007), and Carter (2009), whereas the literature dealing more deeply with the theoretical background (Gutbezahl and Grunwald 1953; Bates et al. 1963; Gelsema et al. 1965, 1966, 1967; Gorina and Kollerov 1974; Mussini et al. 1997) is often complicated and sometimes downright incomprehensible for laymen, including natural history curators and collection managers. Moreover, the proposed theoretical models only roughly approximate the system, and it remains to be tested how realistic they describe the behavior of such mixtures. Empirical studies on the concentration-dependent effect of ethanol (EtOH) on pH measurements are available, but they are generally based on experiments in the presence of buffers or salts (Gutbezahl and Grunwald 1953, Bates et al. 1963, Gelsema et al. 1965, Faraji et al. 2009). This does not represent the situation in natural history collections, where the preservation fluid is generally prepared by mixing EtOH and distilled water (dw).

The issue is, however, very relevant in such collections. A variety of studies have shown that the pH in natural history wet collections varies widely and can reach well into problematic ranges on the acidic as well as the alkaline side (Dingerkus 1982, Cato 1990, Simmons and Waller 1994, Palmer 1996, Waller and Simmons 2003). An extensive screening of EtOH-preserved samples across a wide range of taxa in the collections of two large European museums (Naturhistorisches Museum Basel, Zoologische Staatssammlung

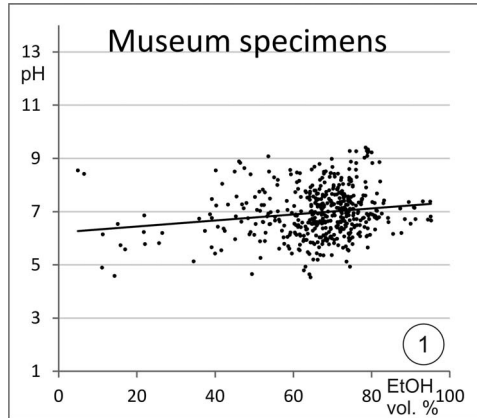


Figure 1. pH and EtOH concentration values obtained from 480 specimen jars from two large museum wet collections (Naturhistorisches Museum Basel, Zoologische Staatssammlung München), covering a broad range of zoological taxa (% EtOH average = 66, standard deviation = 8; pH average = 7.0, standard deviation = 1.3). Trend line computed with Microsoft Excel (2010) (not significant, slope 1%,  $r^2 = 0.0263$ ).

München) revealed a range of pH 4.5–9.5 for both institutions, with 14% of the samples ranging at pH 6 or below and another 14% ranging at pH 8 or above (Fig. 1; Kotrba and Golbig 2009).

The data seems to indicate a very substantial variation, undesirable if not unacceptable in a scientific collection. Preservative solutions should be slightly acidic to provide protection against bacteria and mold (Simmons 2014). Acidic conditions below a pH of 6.4, however, can cause decalcification of bony structures and otoliths, hardening of specimens, as well as protein embrittlement and dissociation, and alkaline conditions substantially above a pH of 7.0 can cause clearing of soft tissues, as proteins and lipids are leached from the specimens. The effects of these acidic and alkaline pH ranges are detailed in the literature, for example in Moore (2002), Hargrave et al. (2005), Kotrba and Golbig (2009), Carter (2009), Simmons (2014).

If pH measurements in hydroalcoholic solutions are difficult and error-ridden, then how feasible is it to monitor the pH in natural history collections for curatorial purposes? Reassuringly, Bates et al. (1963, p. 1833) found that “the response of the glass electrode is often substantially unimpaired at solvent compositions below about 90 wt.% alcohol.” They caution however, that the pH numbers read from the instrument, are “subject to no simple, clear interpretation in terms of chemical equilibrium.” Other authors state that if the solvent background remains constant, then pH measurements in aqueous EtOH solutions are reasonably reproducible and usable within studies looking into acidity changes (Frant 1995, Carter 2009). Ideally, the solvent background should be constant in scientific collections. In reality, however, it can show substantial variation just as the pH does. The values in Figure 1 are plotted against the EtOH concentration measured in the respective samples, showing that the concentration varies strongly, ranging from 5–95 volume % (vol%). Is it possible to estimate the extent and relative importance of systematic errors introduced by the variable and sometimes high EtOH concentration without engaging in the dreaded theoretical and mathematical background? After all, for curatorial purposes even a coarse approximation would be helpful.

Table 1. Sample setup for the five test trials.

Trial	Level	Sample
I	0	10 ml of 0, 10, 30, 50, 70, 80, 90, 96, and 99.8 vol% EtOH in dw
II	0	10 ml of 0, 10, 30, 50, 70, 80, 90, 96, and 99.8 vol% EtOH in dw
	1	Level 0 samples (10 ml) + 1 ml AcOH
	2	Level 1 samples (11 ml) + 1 ml AcOH
III	0	10 ml of 0, 10, 30, 50, 70, 80, 90, 96, and 99.8 vol% EtOH in dw
	1	Level 0 samples (10 ml) + 30 $\mu$ l KOH
	2	Level 1 samples (10.03 ml) + 20 $\mu$ l KOH
IV	0	10 ml of 0, 10, 30, 50, 70, 80, 90, 96, and 99.8 vol% EtOH in dw, 10 ml mw
	0A	Subsamples drawn from level 0 (2.6 ml) + 8 ml mw
	1	Rest of level 0 samples (7.4 ml) + 1 ml AcOH
	1A	Subsamples drawn from level 1 (2.6 ml) + 8 ml mw
	2	Rest of level 1 samples (5.8 ml) + 1 ml AcOH
	2A	Subsamples drawn from level 2 (2.6 ml) + 8 ml mw
V	0	10 ml of 0, 10, 30, 50, 70, 80, 90, 96, and 99.8 vol% EtOH in dw, 10 ml mw
	0A	Subsamples drawn from level 0 (2.6 ml) + 8 ml mw
	1	Rest of level 0 samples (7.4 ml) + 1 ml KOH
	1A	Subsamples drawn from level 1 (2.6 ml) + 8 ml mw
	2	Rest of level 1 samples (5.8 ml) + 1 ml KOH
	2A	Subsamples drawn from level 2 (2.6 ml) + 8 ml mw

The present study takes an empirical approach to look into the effect of EtOH on pH measurements in dependence of the EtOH concentration. The objective was to single out this effect from other variables affecting the fluids in natural history wet collections, such as substances leached from specimens or labels. For this purpose, a series of trials with variable EtOH concentrations in dw was set up and measured, modeling the samples and procedures in natural history wet collections, albeit without the biological specimens or labels. To expand the range of measurements into the acidic and alkaline regime, standardized quantities of acidic or alkaline solutions were added in some of the trials. Moreover, for comparison, some measurements were conducted in samples diluted with natural mineral water. In the course of the study it emerged that the use of dw without adding any buffers or salts poses considerable problems regarding the reproducibility of the measurements and their interpretation. The discussion addresses these as well as other problematic aspects of dw with respect to the treatment of biological samples.

The evaluation and discussion intends to illustrate the empirical results in an intuitively comprehensible way without reaching deeply into physico-chemical theory and the respective mathematical computations. Nevertheless, it is based on a determined struggle with the respective literature.

## METHODS

### *Experimental Design*

The study is based on five trials (Table 1), which were set up and run separately, some of them on different days. The identical setup for all trials consisted of a series of 10-ml samples of rising EtOH concentration (nominal concentrations 0, 10, 30, 50, 70, 80, 90, 96, and 99.8 vol%) in dw. The dw was obtained from a GFL Water Still 2104 for double distillation, which purportedly produces dw with extremely low conductivity and a pH of 5.5 (professional test by Institut Fresenius Chemische und Biologische Laboratorien GmbH,

Taunusstein-Neuhof, Germany, 1991, pers. comm.). After setting up the series, the initial pH and EtOH concentration (referred to as level 0) was measured. In trials IV and V the sample series was extended with a second 0% EtOH sample of commercial mineral water (mw, natural mineral water “Prix garantie” by Coop, containing 597 mg/L sulfate, 221 mg/L calcium, 254 mg/L hydrogen carbonate, 65.4 mg/L magnesium, 4.3 mg/L sodium, 3.3 mg/L chloride, 2.0 mg/L fluoride, < 0.1 mg/L nitrate) instead of dw. In these trials the 2.6 ml subsamples drawn from each sample (level 0) into the measuring chamber of the concentration meter were not returned to the original samples, but instead added to 8 ml of mw. The pH of the resulting solution (level 0A) was measured after thorough mixing. Trials II–V were continued, all samples within a given trial receiving the same treatment with two consecutive additions of stock solutions. In trials II and IV 1 ml of acetic acid (AcOH) stock solution was added each time. In trial III the addition was 30  $\mu$ l of potassium hydroxide (KOH) stock solution (level 1) and additional 20  $\mu$ l (level 2). In trial V, 1 ml of KOH stock solution was added each time. Unfortunately, the concentration of the acidic and alkaline stock solutions was not recorded, but this is of little relevance for the discussed results of this study. After each addition, the samples were thoroughly mixed and the pH measured. In trials IV and V, subsamples were drawn, diluted with mw, and measured as described for level 0 (levels 1A and 2A).

### *Measurements*

All measurements were conducted at room temperature. The pH measurements were taken using a Mettler Toledo SevenGo pH meter with InLab 413/2M/SG electrode which has a PEEK shaft, uses an ARGENTHAL<sup>TM</sup> reference system and XEROLYT<sup>®</sup> polymer reference electrolyte. According to the manufacturer this electrode is particularly insensitive to contamination and its solid electrolyte reduces the amount of aqueous electrolyte, which might enter the system and interfere with the measurements. Its easy use also contributes to the reproducibility of the measurements. The electrode was calibrated with pH 4.01, 7.01, and 10.01 buffer solutions (Hanna<sup>®</sup> Instruments HI 70004, 70010, and 70007). It was rinsed in dw between each reading and frequently regenerated in neutral buffer for several minutes with subsequent recalibration. Because the values tended to drift (especially in the neutral range) all measurements were taken 60 sec after immersion for better comparability. All EtOH concentrations are given in vol% unless otherwise indicated. In the text, the samples are referred to by their nominal concentrations. For the illustrations and computations more exact concentration values were used. When water and EtOH are mixed, the resulting volume is smaller than the sum of the original volumes (Waller and Strang 1996). To avoid respective systematic error, the level 0 EtOH concentration of each sample was measured with an Anton Paar DMA 35 N portable concentration meter. Flushing the measuring chamber twice with the sample before taking the reading ensured that the recorded value is the true concentration of the sample at the start of the trial. Additions of the AcOH and KOH stock solutions change the density of the sample, thus introducing a considerable error in such concentration measurements. The level 1 and 2 EtOH concentrations were therefore calculated from the measured level 0 concentration and the expected dilution by the respective sample treatment.

### *Tables, Computations, Diagrams*

The results for the five trials are summarized in Table 2. Tables and diagrams were generated and computations performed with Microsoft Excel 2010. Diagrams were edited and plates arranged with CorelDRAW X5.

## RESULTS

The resulting data for all trials and levels are summarized in Figures 2–6. For most of the series, the resulting curves are fairly regular (illustrated in black). Only two of them show considerable irregularities, likely due to drifting or erroneous readings (Fig. 6, illustrated in grey). They are excluded from the following evaluation and discussion.

The level 0 curves of all trials theoretically refer to identical series of samples with rising EtOH concentrations in dw without any further additions. To facilitate comparison, all level 0 curves are combined in Figure 7. Apart from a considerable vertical spread, they are obviously similar and two of them are almost identical (trials II and IV). In the 0% EtOH samples the pH ranges from 5.4 to 7.5, indicating a considerable variation in the dw component alone. With rising EtOH concentration, the curves slightly converge, and four of the five meet in the range between pH 7.0 and 7.4 at 99.8% EtOH. The common shape characteristics are a pH drop in the 0% to 30% EtOH range that is complemented by a rise in the longer part of the curves between 30% and 99.8% EtOH. The minimum is reached in the 30% sample or, once, the 10% sample, the true minimum of the curves most likely lying between the chosen concentrations. In some of the curves the slope increases in the range above 90% EtOH. Between 30% EtOH and 90% EtOH, the most acidic of the level 0 curves, which is also the steepest, can roughly be approximated by a 2.5% rising slope (Fig. 7). For the other level 0 curves, this slope is less steep, approximately 1.5% in the least acidic one and intermediary in the others.

The other lines in Figures 2–6 illustrate the pH readings after the addition of one or two quantities of stock solution. Due to the effected dilution, these curves are shorter than the level 0 curves. To facilitate comparison, the level 1 and 2 curves are combined in Figure 8.

For trials II and IV, which received the same additions of acidic stock solution, the results are almost identical. Whereas the first addition is reflected by a strong shift into the acidic regime, the second produced only a small additional shift into the same direction. Similarly, in trial III, the first addition of alkaline stock solution is reflected by a strong shift into the alkaline regime, whereas the second produced only a small additional shift in the same direction. In trial V the general trend is similar, but the curves are very irregular.

Like the level 0 curves, the level 1 and level 2 curves exhibit a pH rise between 30% and 99.8% EtOH. In trials II and IV, which received additions of acidic stock solution, the slope levels off in the lower concentration range, and there is a minute initial drop between the 0% EtOH and the 10% EtOH sample, i.e., the first sample in which any EtOH was added. Between 30% and 80% EtOH, the slope is approximately 2.5% for the level 1 curves and about 2% for the level 2 curves of these trials. In trial III, which received additions of alkaline stock solution, the respective curves show an approximately linear rising slope of roughly 2% throughout the entire concentration range.

Figures 9–14 illustrate the three levels of trials IV and V separately, including the level 0A, 1A, and 2A subsamples diluted with mw and the additional 0% EtOH samples with mw instead of dw. The readings for the additional level 0 samples of pure mw are pH 7.7 for both trials (empty circles in Figs. 9 and 10). This shows that the electrode was equally calibrated for both trials, and that the shift between the rest of the level 0 curves of these trials (full circles in Figs. 9 and 10) is therefore not due to a systematic error of the electrode. Comparison of the 0% EtOH dw samples in levels 1 and 2 with the respective mw samples (empty circles in Figs. 11–14) shows that the shift effected by addition of AcOH or KOH is considerably smaller in the mw samples.

Due to the effected dilution, the level A curves only span the concentration range of 0–25% EtOH. All of them are more or less linear, with a rise toward higher concentrations.

Table 2. Results for the five test trials (for sample setup see Table 1).

Level 0			Level 0A		Level 1		Level 1A		Level 2		Level 2A	
nominal % EtOH	measured % EtOH	pH	calculated % EtOH	pH	calculated % EtOH	pH	calculated % EtOH	pH	calculated % EtOH	pH	calculated % EtOH	pH
Trial I												
0.00	1.00	7.24										
10.00	12.90	6.85										
30.00	31.90	6.65										
50.00	51.60	6.97										
70.00	70.70	7.25										
80.00	80.40	7.38										
90.00	89.60	7.47										
96.00	94.40	7.66										
99.80	98.50	8.50										
Trial II												
					1 ml AcOH added				another 1 ml AcOH added			
0.00	2.80	6.21			2.55	3.54			2.33	3.39		
10.00	11.20	5.85			10.18	3.53			9.33	3.38		
30.00	35.80	5.70			32.55	3.83			29.83	3.65		
50.00	53.20	5.99			48.36	4.07			44.33	3.87		
70.00	71.70	6.26			65.18	4.60			59.75	4.27		
80.00	81.60	6.54			74.18	4.81			68.00	4.43		
90.00	90.50	6.87			82.27	4.95			75.42	4.61		
96.00	95.60	7.15			86.91	5.22			79.67	4.76		
99.80	99.30	7.27			90.27	5.39			82.75	4.89		
Trial III												
					30 $\mu$ l KOH added				another 20 $\mu$ l KOH added			
0.00	1.10	7.48			1.10	11.22			1.09	11.41		
10.00	13.70	6.39			13.66	11.42			13.63	11.66		
30.00	32.30	5.92			32.20	11.70			32.14	11.90		
50.00	52.80	6.49			52.64	12.04			52.54	12.18		
70.00	72.10	6.41			71.88	12.47			71.74	12.53		
80.00	81.20	6.51			80.96	12.74			80.80	12.79		
90.00	90.00	7.11			89.73	12.99			89.55	12.56		
96.00	94.90	6.89			94.62	13.07			94.43	12.99		
99.80	98.60	7.24			98.31	13.17			98.11	13.16		
Trial IV												
			Subsample diluted with mw		1 ml AcOH added		Subsample diluted with mw		another 1 ml AcOH added		Subsample diluted with mw	
mw	0.00	7.67	—	—	0.00	5.67	—	—	0.00	4.4	—	—
0.00	0.80	6.45	0.20	7.59	0.70	3.68	0.17	6.83	0.60	3.39	0.15	6.10
10.00	10.60	5.76	2.60	7.55	9.34	3.66	2.29	6.78	7.96	3.37	1.95	6.14
30.00	31.20	5.73	7.65	7.63	27.49	3.80	6.74	6.86	23.44	3.47	5.75	6.18
50.00	52.00	5.86	12.75	7.64	45.81	4.03	11.24	6.89	39.07	3.73	9.58	6.24
70.00	72.00	6.27	17.66	7.71	63.43	4.62	15.56	6.89	54.10	4.07	13.27	6.34
80.00	82.00	6.61	20.11	7.81	72.24	4.86	17.72	6.97	61.61	4.20	15.11	6.34
90.00	91.20	6.88	22.37	7.74	80.34	5.24	19.71	6.95	68.53	4.44	16.81	6.34
96.00	96.10	6.93	23.57	7.80	84.66	5.39	20.77	6.98	72.21	4.55	17.71	6.39
99.80	99.70	7.36	24.45	7.86	87.83	5.45	21.54	7.05	74.91	4.64	18.38	6.40

Table 2. Continued

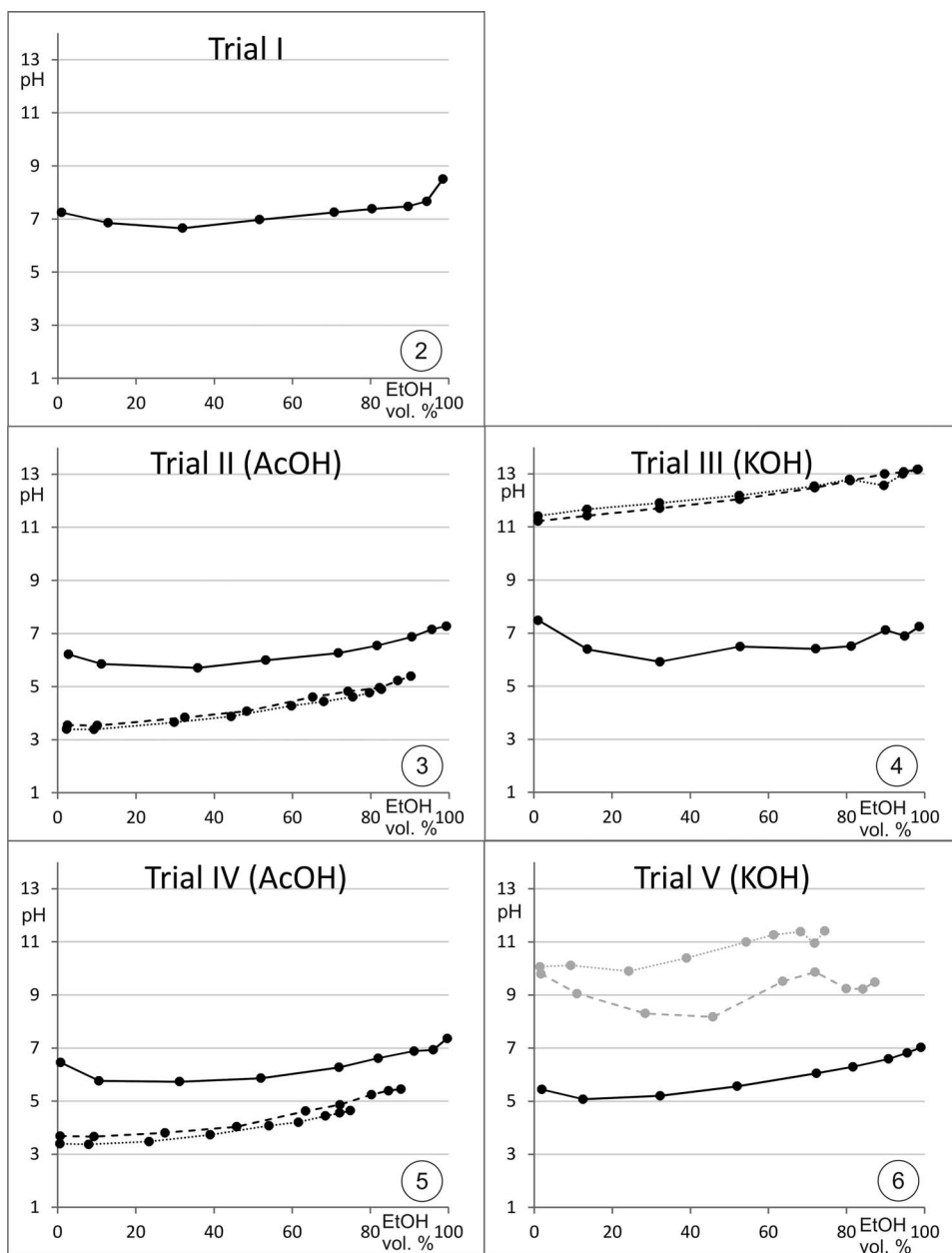
Level 0			Level 0A		Level 1		Level 1A		Level 2		Level 2A	
nominal % EtOH	measured % EtOH	pH	calculated % EtOH	pH	calculated % EtOH	pH	calculated % EtOH	pH	calculated % EtOH	pH	calculated % EtOH	pH
			Subsample diluted with mw		1 ml KOH added		Subsample diluted with mw		another 1 ml KOH added		Subsample diluted with mw	
Trial V												
mw	0.00	7.65	—	—	0.00	8.38	—	—	0.00	8.74	—	—
0.00	2.00	5.44	0.49	7.69	1.76	9.79	0.43	7.98	1.50	10.06	0.37	7.81
10.00	12.50	5.07	3.07	7.79	11.01	9.05	2.70	7.89	9.39	10.11	2.30	7.75
30.00	32.30	5.20	7.92	7.71	28.45	8.30	6.98	7.98	24.27	9.89	5.95	7.78
50.00	52.00	5.56	12.75	7.75	45.81	8.17	11.24	8.00	39.07	10.39	9.58	7.86
70.00	72.30	6.05	17.73	7.90	63.69	9.51	15.62	8.01	54.33	10.99	13.33	7.86
80.00	81.70	6.29	20.04	7.83	71.97	9.86	17.65	8.10	61.39	11.26	15.06	7.91
90.00	90.80	6.59	22.27	7.88	79.99	9.24	19.62	8.16	68.23	11.38	16.73	7.98
96.00	95.60	6.82	23.45	7.98	84.22	9.22	20.66	8.10	71.83	10.95	17.62	7.93
99.80	99.10	7.02	24.31	7.86	87.30	9.48	21.41	8.12	74.46	11.41	18.26	7.94

They reflect neither the pH drop in the 0% EtOH to 30% EtOH range of the original level 0 curves, nor the large irregularities of the original level 1 and 2 curves of trial V. To facilitate comparison, all level A curves are combined in Figure 15. The vertical shift among level 0A, level 1A, and 2A of trial IV reflects on smaller scale the progressive acidification of the original samples. For trial V, the progressive shift of the level A curves into the alkaline regime is present but minute. Graphically elongated trend lines are added to facilitate the comparison of the slopes. The slope of all but the most acidic one is approximately 1%. The most acidic one has a slightly steeper slope of 1.6%.

## DISCUSSION

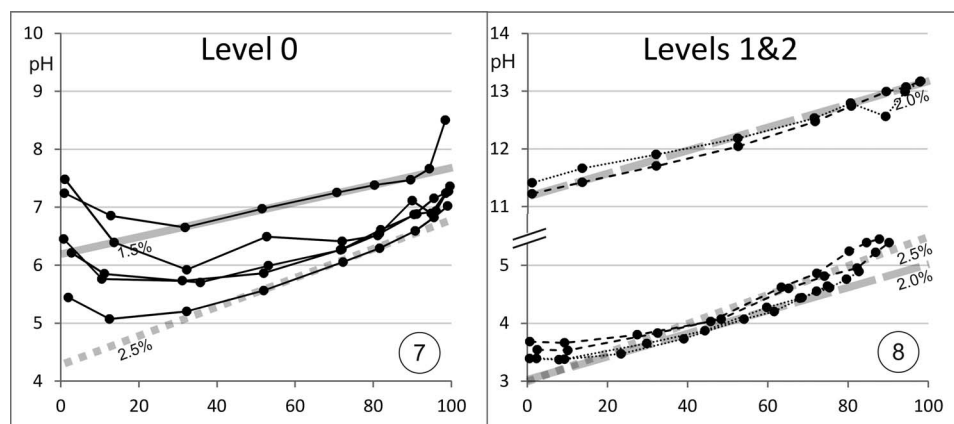
That assigning a pH value to EtOH is not trivial is evident right away from the fact that literature reports different values for pure EtOH, i.e., pH 9.8, 9.55, 7.0, and 7.3, each with its own justification. According to the original definition by Sørensen (1909), the pH is the negative base 10 logarithm of the molar concentration of hydrogen ions or protons ( $H^+$ ). The pH of water at 25°C is 7.0 ( $10^{-7}$  mol/L). Because EtOH dissociates very much less than water, its  $H^+$  concentration is only  $10^{-9.8}$  mol/L, translating into a pH of 9.8. Frant (1995) derives a neutral value of pH 9.55 from an EtOH dissociation constant of  $10^{-19.1}$ . In any case, the  $H^+$  concentration is balanced by the same concentration of the respective dissociated anion (i.e.,  $OH^-$  or  $EtO^-$  resp.). Regarding the acid–base aspect, both water and pure EtOH are therefore neutral. This is often pragmatically translated into a theoretical pH of 7.0, referring to the neutral point of the classical water-based pH scale. The last value, i.e., pH 7.3, is obtained when the pH is determined by measurements by a pH probe (i.e., by comparing the proton activity in the sample with the proton activity in a certain standard buffer), the shift to slightly higher values being caused by several methodological issues, some of which will be briefly discussed below.

Things get worse when EtOH and water are mixed. First, as just mentioned, the dissociation rate of EtOH is much smaller than that of water. In a first approximation, one might speculate that the dissociated  $H^+$  ions present in the water component are simply diluted



Figures 2–6. pH values measured in sample series of rising EtOH concentration in dw without and with the addition of AcOH (trials II and IV) or KOH (trials III and V) stock solution. Solid lines, level 0; dashed lines, level 1 after addition of one quantity stock solution; dotted lines, level 2 after addition of a second quantity of stock solution; grey lines in Figure 6, irregular curves, excluded from evaluation and discussion.





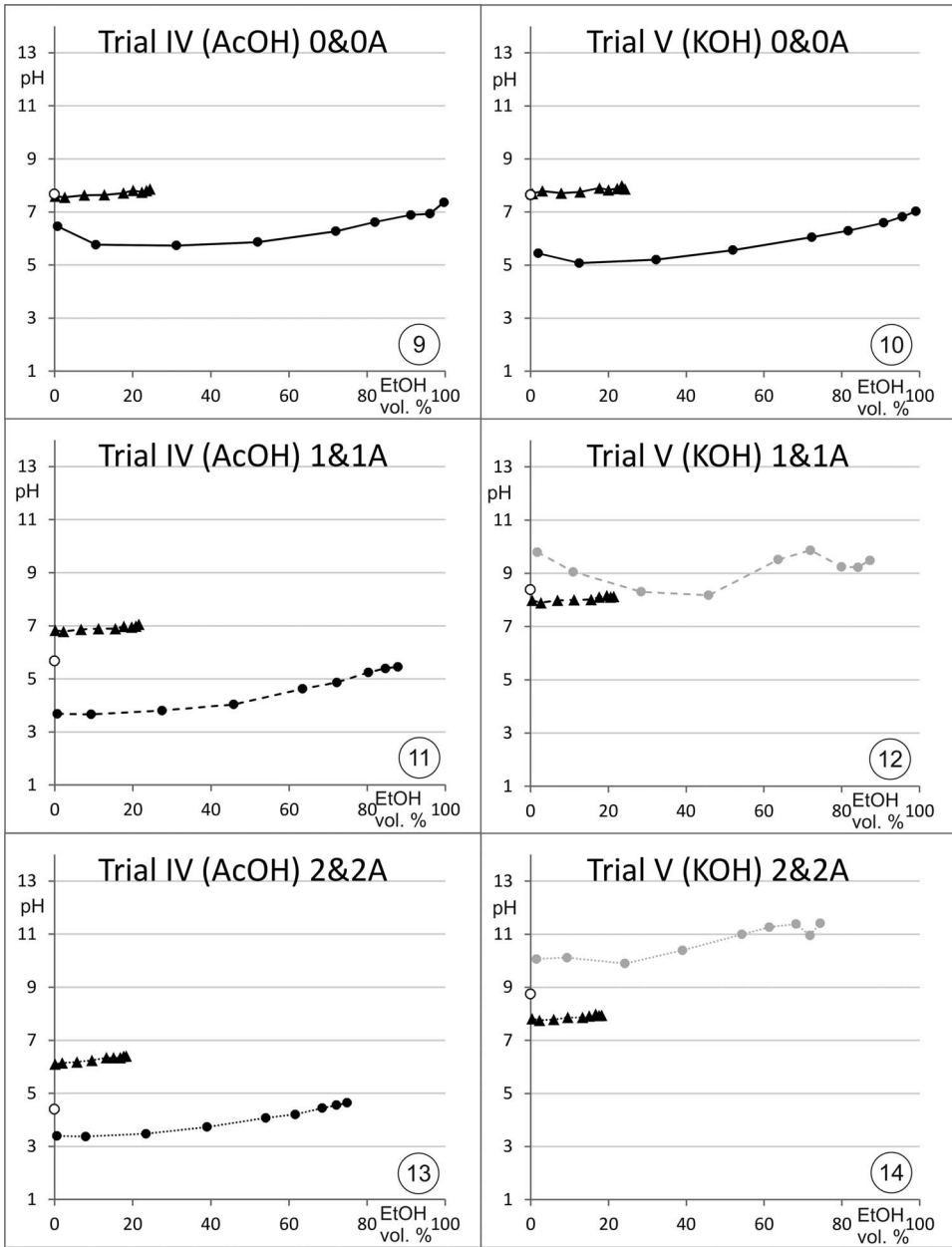
Figures 7–8. pH values in sample series of rising EtOH concentration in dw without and with the addition of AcOH or KOH stock solution. Figure 7: level 0 curves of trials I–V combined for comparison. These curves refer to theoretically identical and theoretically neutral sample series. Figure 8: level 1 and 2 curves of trials II–IV combined for comparison. Solid lines, level 0; dashed lines, level 1 after addition of one quantity stock solution; dotted lines, level 2 after addition of a second quantity of stock solution. Overlaid grey lines indicate slopes for comparison: solid, 1%; dashed, 2%; dotted, 2.5%.

by the EtOH component. This effect would result in the theoretical pH curve illustrated by the dashed line in Figure 16. Note that in this scenario a higher pH, i.e., lower hydrogen ion concentration ( $[H^+]$ ), is the result of dilution and not of a shift in the dissociation equilibrium. It is balanced by a correspondingly lower anion concentration ( $[OH^-]$ ) and therefore constitutes a neutral solution and does not imply a shift from neutral to alkaline conditions.

In reality, matters are more complicated, because the dissociation of EtOH is not negligible and because the dissociation behavior of water and EtOH interact in a nonlinear fashion (Frant 1995). There is a considerable body of literature dealing with various aspects of this highly complicated issue (see Introduction). Some authors have published empirical values, which are summarized in Table 3 and Figure 16 (black solid line). The data of Faraji et al. (2009) were obtained by potentiometric titration of EtOH–water mixtures with  $NaClO_4$  added for maintenance of the ionic strength. Respective data by Gutbezahl and Grunwald (1953) and Gelsema et al. (1965) appear compatible with the findings of Faraji et al. (2009), although obtained with different methods. Notably, up to concentrations of 80% EtOH, the deviation of these data from the simple dilution scenario (dashed line) remains quite small.

Second, EtOH molecules are less polar than water molecules, and thus less apt to “shield” and thus stabilize dissociated ions. This regards the  $EtO^-$  and  $H^+$  ions of the weakly dissociating EtOH itself, but also any other dissociating substances dissolved therein. It is the reason why acids are “less acidic” in EtOH (Frant 1995). Furthermore, acids tend to behave less aggressively in high EtOH concentrations because charged particles resulting from their corrosive reactions are poorly “shielded,” i.e., poorly dissolved in EtOH, and thus cover the reacting surfaces with a protective layer. Note that in this case, a higher pH, i.e., lower  $[H^+]$ , is the result of less dissociation, and not of a shift in the dissociation equilibrium.

Another issue regards the error Delta induced by measuring pH in EtOH–water mixtures using electrodes set up and calibrated with commercial aqueous standard buffers without the respective EtOH component. If the internal electrolyte of a combined pH electrode



Figures 9–14. pH values in subsamples from trials IV and V (Figs. 5 and 6) diluted with mw. Full circles, levels 0–2 (undiluted); empty circles, additional sample with 0% EtOH in mw; triangles, level 0A–2A (diluted subsamples). Solid lines, level 0; dashed lines, level 1; dotted lines, level 2. Grey lines in Figures 12 and 14: irregular curves, excluded from evaluation and discussion.

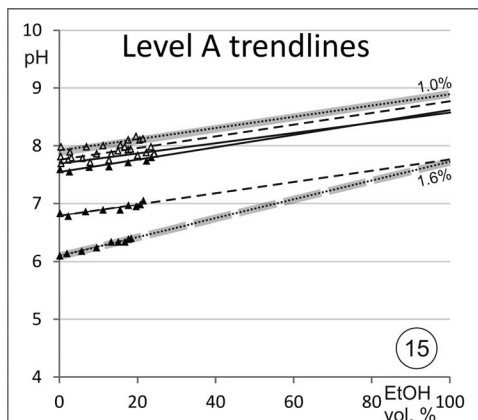
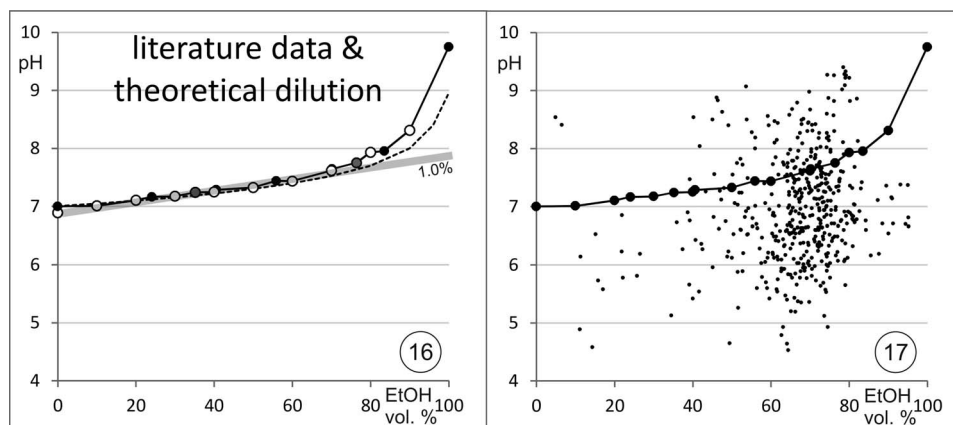


Figure 15. Level A curves of trials IV and V (Figs. 9–14) combined for comparison. Trend lines computed with Excel, graphically elongated to facilitate comparison. Full triangles, trial IV; empty triangles, trial V. Solid lines, level 0A; dashed lines, level 1A; dotted lines, level 2A. Overlaid grey lines indicate slopes to facilitate comparison: solid, 1%; dashed, 2%.

is purely aqueous, this causes an additional electric potential at the junctions between the purely aqueous system and the (partially) organic solution. A number of authors have investigated this effect and published corrective values, which are summarized in Table 3. Because Delta is very small ( $\text{pH} \leq 0.3$ ) for much of the concentration range and only becomes substantial above 90% EtOH, it is disregarded in the present context.

Considering these various, interacting, and overlaying properties of EtOH, it appears almost impossible indeed to predict how variable concentrations of EtOH will affect the



Figures 16–17. EtOH concentration-dependent effect and sample comparisons. Figure 16: empirical data from the literature (Table 3) and theoretical calculation. Empty circles, data of Faraji et al. (2009); black circles, data of Gutbezahl and Grunwald (1953); grey circles, data of Gelsema et al. (1965). Dashed line: theoretical values based on the simplified model that  $[\text{H}^+]$  is diluted by the EtOH component, computed using  $\text{pH}_{\text{theoret}} = -\text{Log}((100 - \text{vol}\% \text{EtOH})/100 * 0.0000001)$ . Overlaid grey line: 1% slope for comparison. Figure 17: comparison of pH and EtOH concentration values from museum wet collections (Fig. 1) with the empirical characteristic line based on published data (Fig. 16).

Table 3. Literature data for pH and Delta in dependence of the EtOH concentration. pH calculated from auto-protolysis constant  $pK_{ap}$  resp.  $pK_s$  using  $pH = 0.5 pK$ . Vol% EtOH marked by asterisk are computed from wt% using <https://rechneronline.de/volumenprozent-massenprozent/>.

Vol% EtOH	pH	Delta	Author
0.0	7.00		Gutbezahl and Grunwald (1953: Table III)
0.0	6.89		Faraji et al. (2009: Table 1, potentiometric titration)
10.0	7.01		Faraji et al. (2009: Table 1, potentiometric titration)
19.7		0.00	Bates et al. (1963)
20.0	7.11		Faraji et al. (2009: Table 1, potentiometric titration)
24.1*	7.17	- 0.03	Gutbezahl and Grunwald (1953: Table III)
30.0	7.18		Faraji et al. (2009: Table 1, potentiometric titration)
35.2*	7.24		Gelsema et al. (1965: Table II)
35.2*		0.11	Gelsema et al. (1966)
38.7*		0.09	Bates et al. (1963)
40.0	7.25		Faraji et al. (2009: Table 1, potentiometric titration)
40.6*	7.29	0.07	Gutbezahl and Grunwald (1953: Table III)
50.0	7.33		Faraji et al. (2009: Table 1, potentiometric titration)
55.9*	7.44	0.17	Gutbezahl and Grunwald (1953: Table III)
55.9*		0.29	Gelsema et al. (1966)
57.9*		0.22	Bates et al. (1963)
60.0	7.44		Faraji et al. (2009: Table 1, potentiometric titration)
70.0	7.62		Faraji et al. (2009: Table 1, potentiometric titration)
70.2*	7.65	0.19	Gutbezahl and Grunwald (1953: Table III)
76.4*	7.75		Gelsema et al. (1965: Table II)
76.4*		0.33	Gelsema et al. (1966)
77.8*		0.20	Bates et al. (1963)
80.0	7.93		Faraji et al. (2009: Table 1, potentiometric titration)
83.5*	7.96	0.11	Gutbezahl and Grunwald (1953: Table III)
88.3*		- 0.03	Bates et al. (1963)
90.0	8.31		Faraji et al. (2009: Table 1, potentiometric titration)
100.0	9.75	- 2.36	Gutbezahl and Grunwald (1953: Table III)
100.0		- 2.91	Bates et al. (1963)

measured pH values based on theoretical deliberations (Frant 1995, Tindall and Dolan 2002). Waller and Simmons (2003, p. 14–15) point out that “a simple, singular interpretation of a pH reading in an EtOH–water solution is not possible” and “a solution with  $pH = 6$  at 70% v/v ethanol may or may not be more acidic than a solution with  $pH = 7$  at 0% v/v ethanol.”

An alternative approach is to describe the phenomenon based on empirical results. The present study had the objective to generate a simple approximative guideline for the interpretation of such data based on our own empirical results as well as data published by previous authors.

For this purpose, trials with samples of variable EtOH concentrations in dw were measured (level 0). In some of the trials, standardized quantities of acidic or alkaline additives were added to expand the range of measurements into the acidic and alkaline regime (levels 1 and 2) and in some of these subsamples were drawn and diluted with mw (level A).

For all these trials, the resulting curves show a rise toward higher EtOH concentrations throughout all or at least the largest, and for curatorial aspects most relevant, part of the concentration range between 30% and 80% EtOH (Figs. 7, 8, 15). The results suggest that, under otherwise identical circumstances, higher EtOH concentrations will yield higher pH readings and that the steepness of the respective slope increases from about 1% to up to

2.5% when acidifying or alkalinizing substances are added. The variation of the slope and shape of the curves, as well as the theoretical background suggest, however, that matters are considerably more complicated and merit a more detailed discussion.

The level 0 curves were designed to generate a kind of “neutral” calibration curve for curatorial purposes. Their major distinctive feature with respect to the available literature is that they regard measurements in EtOH–dw mixtures without any buffers, salts, or other conductivity enhancing substances added. This composition mirrors the preservation fluids most commonly used in natural history wet collections today. Unfortunately, the results did not meet the expectations. The obtained values indicate that the “neutral” level 0 samples were not neutral (and, judged from this, neither are the fluids generally used in our collection jars). Instead, the level 0 curves show a particularly large and a priori unexplained vertical spread, reaching deeply into the acidic regime. Moreover, their strongly curved shape deviates considerably from the other curves in this study, particularly the corresponding level 0A curves, and from comparable literature data (see below). These peculiarities are likely related to the specific properties of dw and will be discussed in that context further below.

All other curves (levels 0A, 1, 1A, 2, 2A), are fairly regular, and the deviations observed between the comparable curves of trials II and IV (AcOH added) are minute, suggesting a fair reproducibility. The higher reproducibility is likely due to the greater conductivity of these samples and, in case of the level A curves, the buffering capacity of the mw.

The level A curves appear particularly interesting, and it is regrettable that, because they were generated by the dilution of subsamples, the results cover only part of the concentration range. The slope of approximately 1%, which characterizes most of the level A curves (Fig. 15), roughly matches the slope of the curve resulting from the published empirical values (Fig. 16).

The combined evidence suggests that the curve based on the published data might be serviceable as an empirical characteristic curve to estimate the magnitude of the EtOH concentration-dependent effect, provided that there is sufficient conductivity and that no larger quantities of acidifying or alkalinizing substances are involved.

In Figure 17, this characteristic curve is combined with the pH values measured in representative samples from two natural history collections (same as Fig. 1). The measured values are rather evenly distributed around pH 7. The majority, however, lie below the empirical “neutral” values. This demonstrates that the actual acidity of such samples easily can be underrated if the concentration-dependent effect of EtOH is not taken into account. Revisiting the example from Waller and Simmons (2003), the suggested characteristic curve implies that a solution with pH 6 at 70% EtOH is indeed more acidic than a comparable solution with pH 7 at 0% EtOH. It can be estimated that to (re)constitute neutral conditions in the 70% EtOH sample, its pH would need to be raised to slightly above pH 7. Moreover, based on the theoretical information above it can be presumed that the 70% sample presently contains more (albeit less dissociated) acid than a 0% EtOH sample (i.e., water) would contain at the same pH of 6. The slope of the characteristic curve might also be used for the direct comparison of samples of different EtOH concentration. For example, a difference of pH 0.6 would be expected between a 50% EtOH and an 80% EtOH sample. If instead, a difference of pH  $-0.2$  was measured, then a larger difference, perhaps closer to pH  $-0.8$ , would have to be explained by other factors, such as substances leaching from specimens or labels.

The suggested characteristic curve can thus be used to consider samples near the neutral range. The slope gets steeper, however (up to 2.5% in our study), when acidifying

or alkalinizing additives are involved. This is evident in the level 1 and 2 curves for trial II–IV (AcOH or KOH added, Fig. 8), as well as the most acidic one of the respective level A curves (Fig. 15), and to a variable degree the level 0 curves (Fig. 7), in which the acidifying component is very likely carbon dioxide (CO<sub>2</sub>) absorbed from the atmosphere and reacting with water (H<sub>2</sub>O) to form carbonic acid (H<sub>2</sub>CO<sub>3</sub>, see below). In these instances, the concentration dependent EtOH effect per se is augmented by effects resulting from the EtOH concentration-dependent dissolution or dissociation of the added components.

In the case of the organic acid, AcOH, the steeper slope results from the fact that, although it dissolves equally in dw and EtOH, it is less dissociated and therefore less acidic at higher EtOH concentrations (see above). Similarly, H<sub>2</sub>CO<sub>3</sub> will be less dissociated in higher EtOH concentrations. KOH, on the other hand, is much less soluble in EtOH than in dw, but all KOH that is dissolved is also dissociated. This is because the bond between the potassium cation (K<sup>+</sup>) and the hydroxide anion (OH<sup>-</sup>) is not covalent but ionic. In this case, the steeper slope of the curves could result from the fact that at higher EtOH concentrations, the dissolved KOH is progressively concentrated in the remaining aquatic component.

The magnitude of such effects and the resulting slope will depend on the chemical nature of the added substances, but the qualitative pattern of increasing pH with increasing EtOH concentration will hold true for the vast majority of additives. The trend could only be inverted if an additive chemically binds EtO<sup>-</sup>, thus effecting the release of additional protons into the medium. Such compounds are very rare, however, and very unlikely to occur in biological samples. Last not least, a variety of complex ionic interactions can be empirically correlated with changes in the ionic strength of a sample. This effect might interfere with the proton activity. These effects are very complex and hard to predict. Thus, they cannot be adequately discussed here.

The Level 0 curves (Fig. 7) differ from all curves discussed so far by a substantial drop in the range between 0% and 30% EtOH. Moreover, the theoretically identical and theoretically neutral level 0 curves in toto show a variable, unexplained, and a priori implausible shift into the acidic regime. These peculiarities are likely related to the level 0 setup involving pure EtOH–dw mixtures without any buffers, salts, or other conductivity enhancing substances added. These particular conditions much complicate the measurements, affect the results, and muddle their interpretation. Distilled water is notoriously problematic with respect to pH measurements due to its low electrical conductivity, i.e., high electrical resistance (Tse 2007, Enzler 2012). Some authors report particular problems with “drifting readings” (Sound and Becker 2007, Yokogawa Electric Corporation 2015); others state that the obtained values will be “somewhat meaningless” (Harris 2017).

Distilled water is also extremely sensitive to contamination because it has little, if any buffering capacity. In trials IV and V, the dw samples were considerably more affected by the addition of AcOH or KOH than the respective mw samples. Even very low levels of contamination, such as with absorbed atmospheric CO<sub>2</sub>, can cause substantial pH changes in dw (Tse 2007, Harris 2017). Enzler (2012) points out that dw tends to absorb CO<sub>2</sub> from the atmosphere, which can lower its pH from about 7 to about 5.8 within a few hours. In the present study, dw was obtained from a water still, whose product, according to professional testing (Institut Fresenius 1991, pers. comm.) settles at pH 5.5. This plausibly explains why the level 0 curves are shifted into the acidic regime. Hargrave et al. (2005, p. 27) similarly observe that “the source of water used to mix the solution influenced the initial pH of the solution, which affected the final pH of the storage fluids across all treatments.” The

variable magnitude of the shift, evident from the pH values of the 0% EtOH samples of level 0 ranging from pH 5.4 to 7.5 (Fig. 7), might be due to variable time intervals passed between distillation and measurements, and correspondingly variable amounts of CO<sub>2</sub> absorbed from the atmosphere. However, the possibility of other contamination or systematic measurement error cannot entirely be excluded. Because the phenomenon likely regards a unidirectional deviation, it cannot be eliminated by averaging.

The pH drop between 0% EtOH and 30% EtOH remains inexplicable. One possible explanation is the observation that the addition of EtOH to water causes a pronounced increase in the solubility of CO<sub>2</sub> (Dalmolin et al. 2006). This, in consequence, might lead to elevated levels of dissociated carbonic acid (HCO<sub>3</sub><sup>-</sup> + H<sup>+</sup>) in the solution. The resulting acidification could be prominent in the ion-poor level 0 conditions, but small compared to the strong acidification effected by the addition of AcOH in trials II and IV, and therefore mostly drowned out on the logarithmic scale of the respective graphs. In trial III, in which the effect does not surface at all, the effect might be neutralized by the addition of large amounts of KOH.

Resolving the nature of this effect could be of considerable relevance for natural history wet collections. If the use of dw really causes acidification of the samples in such a way, then this will be particularly problematic in samples that contain only a small proportion of organic tissue, e.g., single type specimens, and therefore have little buffering capacity. Moreover, in this case it will be impossible to establish a “neutral” pH of 7 or more, by exchanging with, or adding more of, the same kind of preservative.

The problems induced by the use of dw without any additives raise questions about the use of dw in preservation fluids for natural history collections in general. Every naturalist is well aware of the detrimental effects dw has on organic samples. Water influx due to osmotic effects causes swelling or rupturing of the tissues. At the same time various substances are leached from the tissues, a process which can alter the optical, mechanical, and chemical properties of the samples. This is why sophisticated Ringer’s solutions, or at least physiological NaCl solutions, are generally used for the treatment of live tissues as well as for state-of-the-art fixation of scientific samples. Much to the contrary, dw or, more rarely, tap water is used to mix the preservation fluids in natural history wet collections, with tap water generally being considered the poorer choice, because, depending on its origin and method of purification, it can contain a multitude of inorganic and organic components that can cause precipitation or can affect the pH of the sample (Dingerkus 1982; Simmons 1995, 2014; Hargrave et al. 2005; Notton 2010).

The alcohol component of the preservation fluid causes dehydration and a crude fixation of the tissues, thus vaguely counteracting the osmotic water influx which would occur in dw alone. Devoid of any dissolved substances, it does not, however, impede the dissolving and leaching of substances from the samples. Apart from the resulting detriment to the specimen, these substances can also alter the chemical properties of the preservation fluid, such as the pH, either directly or after interaction with oxygen or other dissolved substances.

In summary, dw constitutes an “osmotic disaster” for biological tissues, and a nightmare regarding pH issues. It has an unreliable and generally somewhat acidic pH to begin with and is extremely susceptible to pH changes due to contamination or CO<sub>2</sub> uptake. Moreover, for monitoring the pH, dw constitutes a bigger problem than does the involved EtOH component.

In this context it is also noteworthy that the commonly recommended dilution of samples with dw for the very purpose of measuring the pH might be counterproductive. Although

such dilution will indeed reduce the EtOH effect, it can at the same time considerably alter the pH by introducing the possibly acidic or alkaline pH of the added dw and by causing higher dissociation rates of dissolved substances.

Whether tap or mineral water constitute good alternatives, depends on the chemical specifics. A better option might be to add some salts or buffers to the preservation fluid. Tse (2007) suggests that the addition of a neutral salt such as KCl to distilled water will increase the concentration of dissolved ions and thus improve the reliability of pH measurement.

### CONCLUSIONS

Considering the scientific value of museum specimens and the accumulated effort that goes into their acquisition, processing, and storage, it is certainly unwise to jeopardize their continued availability and quality by neglecting curatorial care over time. Natural history collections therefore need to define appropriate preservation protocols, specifying not only the initial conditions, but also their surveillance and maintenance. This not only concerns the quality and concentration of the alcohol used, but also the water used, the pH, and possibly the osmotic regime of the preservation fluid. The issue is all the more important because many natural history specimens do not undergo any initial fixation.

The large pH deviations found in natural history wet collections (Fig. 1) underline the necessity to monitor the pH in such collections. The present study shows that the difficulties posed for pH measurements by the variable EtOH concentrations in such collections are not so large as to render such measurements futile and are not a sufficient excuse to ignore the issue altogether. Instead, this study suggests how the EtOH concentration-dependent effect could be taken into account for curatorial purposes.

The present study failed, however, to empirically produce a reliable quantitative “calibration curve” for the pH measured by a commercial electrode in EtOH–dw mixtures in dependence of the EtOH concentration. Theoretically it should be possible to obtain a reproducible curve for such an apparently simple and clearly defined system. Practically, this was not successful, because the unstable pH of the dw component was not anticipated and the resulting errors could not be eliminated. It appears that the particularities of dw constitute an important, complicated, and cumbersome problem with far-reaching implications, not only for the present measurements, but also for the curation of natural history wet collections in general.

This important issue urgently requires continued research efforts: (1) to produce empirical “calibration curves” under more reproducible conditions, which ideally should be done individually for any specific laboratory setting and electrode; and (2) to explore the rationales and possibilities of substituting more suitable diluents, such as some kind of Ringer’s solution, for dw in natural history wet collections.

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