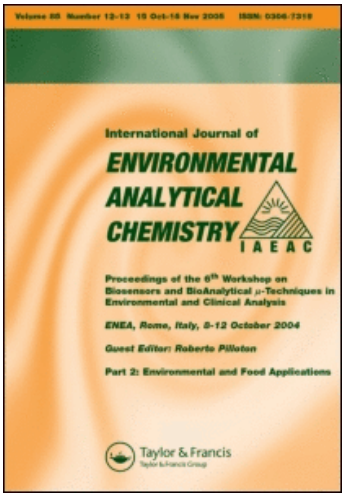

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Preservation of Throughfall Samples by Chloroform and Thymol

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PRESERVATION OF THROUGHFALL SAMPLES BY CHLOROFORM AND THYMOL

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Chloroform (trichloromethane) and saturated thymol (5-methyl-2-[1-methyl ethyl]phenol) were used as biocides to preserve synthetic and real throughfall water samples for up to 50 days. Three replicate sealed sample bottles were kept in a cold room in the dark at 2°C to simulate laboratory storage conditions, and three replicate samples were kept in the dark unsealed, but covered, in a greenhouse at an average of 20°C to simulate storage in the field.

Both additives maintained the pH, conductivity and NH_4^+ content of both synthetic and real throughfall samples for 50 days in the cold room while only thymol could maintain concentrations beyond 14 days in the greenhouse. In samples without biocide there were some changes of chemical composition after 7 days, even in the cold room. The loss of effectiveness of chloroform at the higher temperature was caused by evaporation from the unsealed bottles, and resulted in mould growth and marked loss of NH_4^+ from solution.

The use of a saturated solution of thymol as a preservative is recommended where throughfall is sampled at weekly (or longer) intervals, to prevent loss of organic acids and NH_4^+ ions, and maintain sample pH prior to collection and laboratory analysis. Without such precautions, measurements of NH_4^+ and NO_3^- in throughfall are likely to be unreliable indicators of the net flux of N below a forest canopy.

KEY WORDS: Throughfall, ammonium, biocide, preservation.

INTRODUCTION

Large changes in the H^+ ion activity ("free acidity" measured as pH) of precipitation may occur within a few hours after sample collection[1]. In view of the changes in pH and in the concentrations of other components in collected precipitation, Galloway and Likens[2] recommended that event samples be analyzed immediately after collection, but this condition cannot be met when, for logistical or economic reasons, composite precipitation samples are collected over periods of a week or longer. An essential requirement, therefore, for any rain water composition study based on sampling periods longer than that of individual events is the selection of a biocide which prevents the biological degradation of collected samples.

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These problems are well know for rain samples, which in general have relatively small concentrations of dissolved organic carbon and are relatively uncontaminated by living organisms. Throughfall, however, which has passed over the canopy surface of a forest or other vegetation, may have gained large concentrations of water-soluble organic compounds, and may contain suspended microorganisms washed from leaf and branch surfaces. The potential for biological activity is therefore greatly increased, and the consequent demands on an effective biocide are more stringent than for rain. The use of methyl mercury as a biocide for throughfall samples has been reported[3], and laboratory experiments[4] have shown the importance of a biocide in controlling microbial uptake of nitrogen-containing ions.

Recent measurements of the chemical composition of throughfall, and the relationship to the composition of incident precipitation, arose through an interest in the ability of plant canopies, particularly forests, to 'neutralise' the acidity of polluted rain. The acidity of throughfall may be greater or less than that of the rain falling on the canopy, as ions are exchanged at plant surfaces, and dry-deposited acidic or alkaline substances are washed off[5]. Increases in acidity, attributed to the wash-off of acidic gases and particles, were often associated with increases in sulphate deposition below forest canopies, and led to the use of measurements of sulphate in throughfall to estimate total sulphur deposition to forests. The assumption that the additional sulphur in throughfall is derived solely from the dry deposition of SO_2 and sulphate particles is an area of active debate[6], but there has been no suggestion that the loss of sulphate ions from throughfall samples as a result of biological activity is a confounding factor in the argument.

The apparent success of this technique for sulphur, using throughfall measurements to estimate total deposition, has prompted the use of such measurements to estimate deposition of nitrogen to forests, both as NH_4^+ and as NO_3^-. Apart from the possibility of uptake of these ions by foliage, measurements of NH_4^+ and NO_3^- concentrations in throughfall samples which have been in the field for days or weeks prior to collection are unlikely to be reliable if micro-organisms have assimilated these nitrogen-containing ions. It is unlikely that throughfall measurements can be used directly to estimate deposition of nitrogen to forests, as for sulphur, because N-containing ions and molecules are consumed and produced in the canopy, both by the tree and by epiphytic micro-organisms. However, estimates of residual N in throughfall are useful in assessing the extent of such canopy processing, as part of a better understanding of the cycling of N in forests. The use of biocides in throughfall samples is not well documented, and often does not appear to be part of the sampling protocol.

Measurements of N-containing ions and molecules in throughfall samples which have not been adequately preserved are unlikely to be useful in estimating this component of the N cycle.

This study was undertaken to assess the biocidal effectiveness of chloroform and thymol on throughfall water samples stored in darkness under two different regimes, simulating:

(i) storage under ideal laboratory conditions, and

(ii) long-term sampling in the field.

METHODS AND MATERIALS

Ten litres of synthetic throughfall were prepared from analytical grade reagents without further purification, containing H^+ (50 μM), Na^+ (80 μM), K^+ (30 μM), Ca^{2+} (20 μM),

Mg^{2+} (20 μM), NH_4^+ (40 μM), Cl^- (110 μM), NO_3^- (40 μM), SO_4^{2-} (65 μM). Acetic acid (20 μM) was added to simulate the organic acid content of throughfall. This increased the H^+ ion concentration by 5 μM through dissociation. An aliquot of 450 mL of synthetic throughfall was put in each of 18 polyethylene bottles (500 ml capacity), 6 containing 5 ml chloroform, 6 containing about 140 mg thymol[7] and the remaining 6 with no biocide.

Real throughfall water was collected under 20-year old lodgepole pine (*Pinus contorta* Mill.) trees at Bush Estate, Penicuik, Scotland. A polyethylene gutter, 10 m x 0.5 m, was constructed under the canopy 0.5 m above ground, and drained into two 10-litre polyethylene bottles fitted with nylon net to remove large particles. A sample of throughfall following heavy overnight rain was used to prepare the experimental sub-samples for analysis. 18 sub-samples, each containing 450 ml of the sampled throughfall, were prepared as above, with and without biocide.

Nine synthetic samples (3 with chloroform, 3 with thymol and 3 without biocide) and nine throughfall samples (3 with chloroform, 3 with thymol and 3 without biocide) were sealed and stored in darkness in a cold room at 2°C to simulate laboratory storage of collected samples prior to chemical analysis. The remaining samples (9 synthetic and 9 throughfall) were stored unsealed, but covered to exclude dust, in a greenhouse at 20 ±3°C, to simulate prolonged sampling periods in the field. Bottles were enclosed in a black plastic bag, with the neck of the bottle exposed to light, as would occur in the field.

Measurements of pH, conductivity and NH_4^+ content were made on all 36 samples before starting the experiment and after 1,7,15,29 and 50 days of storage. The pH was measured using a temperature-compensated combined electrode calibrated using dilute sulphuric acid standards at pH 4 and pH 3[8]. Hydrogen ion activity ($a_{H^+}/\mu M$) was calculated from pH directly as $10^{(6-pH)}$, but for simplicity is referred to hereafter as hydrogen ion (H^+) concentration. Conductivity was determined using a temperature-compensated conductivity cell, calibrated using a solution of 0.1 M KCl. Samples were brought to room temperature before measurement to minimise temperature effects. Concentrations of NH_4^+ were measured by continuous flow colorimetry following the method of Sutton[9], utilizing the reaction between dichloroisocyanurate and salicylate, catalysed by nitroprusside to form a blue indophenol dye in the presence of NH_3. Calibration was by means of solutions of Analytical Grade NH_4Cl in deionized water, serially diluted to give a range of 0, 50, 100, 200 and 500 μg NH_4^+ $litre^{-1}$, with the response fitted to a quadratic curve.

There were initial problems with the analysis of thymol-treated solutions for anion composition by ion chromatography. This side-effect of the biocide was overcome by filtering an aliquot of the thymol-treated samples through a glass-fibre pre-filter, an 0.45 μm cellulose membrane filter and a solid-phase extraction cartridge (C_{18}-silica: Waters Sep-Pak®), connected in series, prior to analysis.

Significant differences among the different biocide treatments (no biocide, thymol, chloroform) were assessed by one-way analysis of variance at each measurement date for each set of samples (synthetic or real throughfall) and storage conditions (coldroom or greenhouse). Changes over time were assessed by fitting a straight line to the data and testing for significant differences from zero slope.

RESULTS

Measurements made on all 36 samples at the start of the experiment showed no significant differences among the sub-samples used for each combination of biocide

treatment and storage conditions. The average concentrations of hydrogen and ammonium ions, and of conductivity, are given in Table 1. The standard deviations derived from these analyses may be used to estimate the precision of the analytical methods used. In all cases the standard deviation (n=18) was less than 6% of the mean, with the largest uncertainty arising from estimation of hydrogen ion concentrations from pH measurements of the synthetic samples. The theoretical concentrations for the synthetic solution are also shown, and agree with the measured values to within the measurement uncertainty (± 2 std. dev.).

Table 1 Initial concentrations of synthetic and throughfall solutions, from measurements of each of 18 replicates of each type.

Measurement	*Synthetic*		*(theoretical)*	*Throughfall*	
	mean	*std.dev.*		*mean*	*std.dev.*
pH	4.254	0.023		3.761	0.016
H^+(μM)	55.7	2.9	55	173	7
NH_4^+(μM)	36.8	1.6	40	354	13
Conductivity	56.9	0.6	54.7	346	2

Coldroom—sealed samples

The concentrations of H^+ and NH_4^+ and the conductivity of synthetic samples stored in the coldroom at 2°C are shown in Figure 1. Significant differences among solution treatments on a given date are shown by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Concentrations of H^+ decreased, even in thymol-treated samples, to c. 50 μM after 14 days. As this was the concentration of strong acid originally present, this result suggests that acetic acid was slowly lost from solution, either by adsorption to, or diffusion through, the container walls. There were no significant differences between samples treated with thymol or with chloroform, except for NH_4^+ after 50 days, and even here the difference was within the uncertainty of the analytical method. Measurements of conductivity varied the most over time, possibly as a result of calibration differences on different sampling dates.

Results for the throughfall samples stored in the coldroom are shown in Figure 2. Again, there were no significant differences between the two sets of samples treated with biocide, but significant differences after 14 days in the untreated samples. Concentrations of NH_4^+ increased by c. 100 μM over the 50 days, matched by a similar decrease in H^+ concentrations. This led to a significant decease in conductivity, because of the greater specific conductivity of the H^+ ion compared with the NH_4^+ ion. Calculation of the residual conductivity, by subtracting the theoretical conductivity of H^+ and NH_4^+ from the measured value, showed that there was no significant difference in concentrations of other ions between biocide-treated and untreated samples over the 50-days period. There were also no significance differences among sample types for the sum of H^+ and NH_4^+ concentrations.

The changes in untreated and chloroform-treated samples relative to thymol-treated samples are shown in Figure 3, which more clearly illustrates the temporal changes, as small differences in absolute concentrations between sampling dates, caused by calibration changes in the analytical methods between sampling dates, are effectively eliminated.

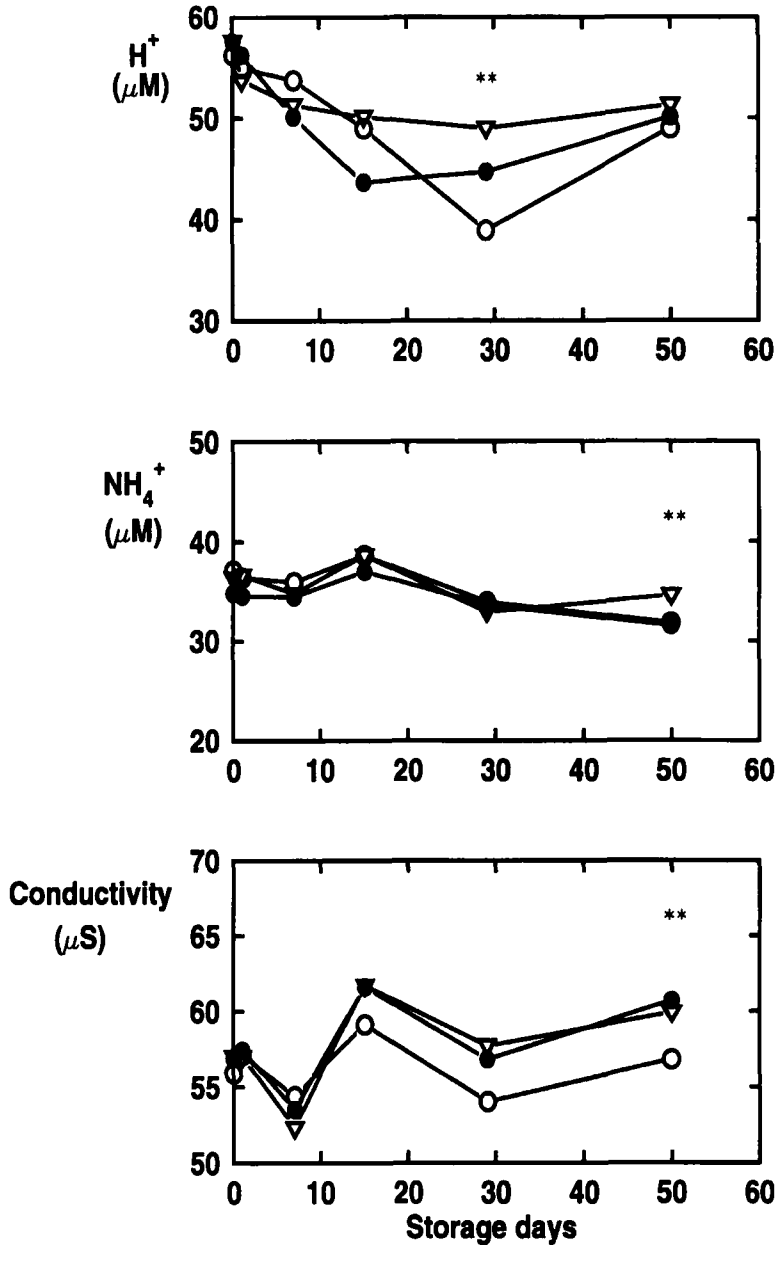

Figure 1 Concentrations of H^+, NH_4^+ and electrical conductivity of synthetic throughfall solutions, containing thymol or chloroform as biocide or no biocide, stored in sealed bottles in a coldroom at 2°C for up to 50 days. Significant differences among biocide treatments at each sampling time, using one-way ANOVA, are denoted by asterisks (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). There were 3 replicates per treatment.

Open circles: no biocide added
Open triangles: thymol added
Filled circles: chloroform added.

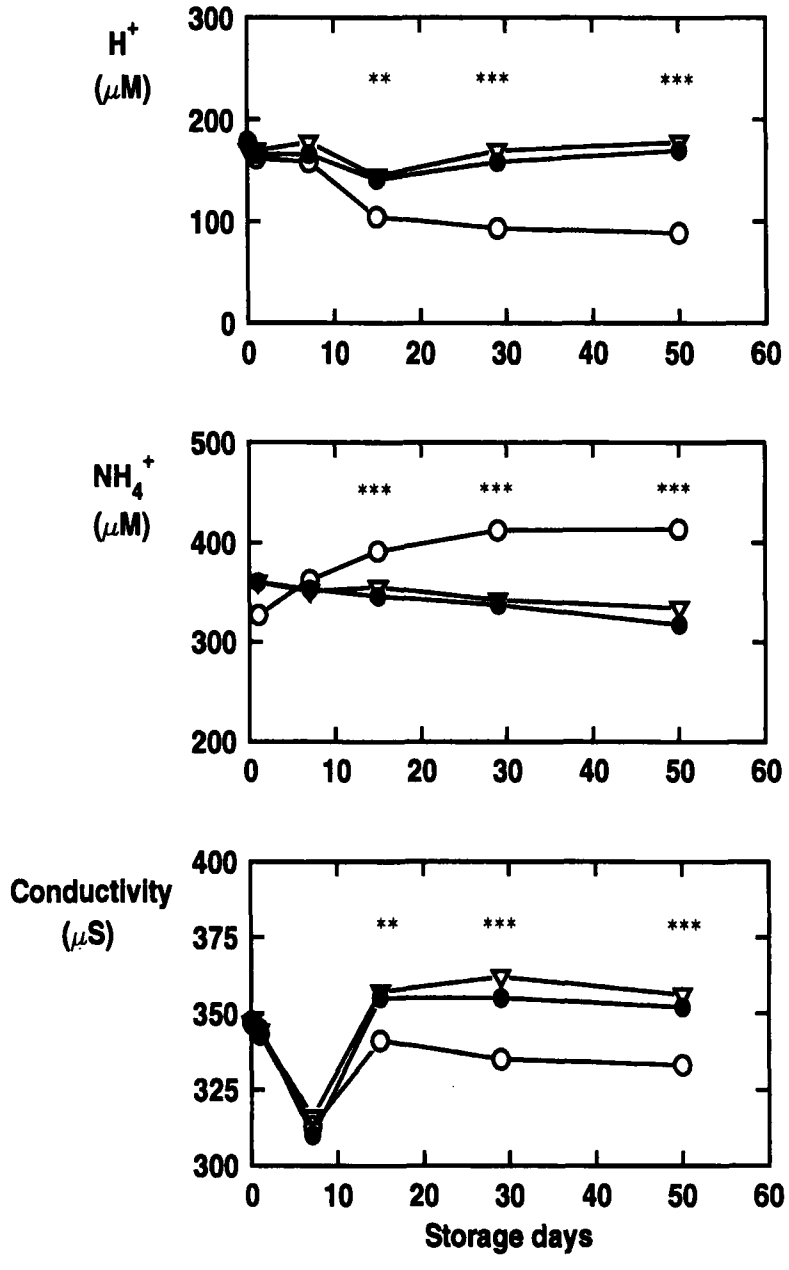

Figure 2 Concentrations of H^+, NH_4^+ and electrical conductivity of real throughfall solutions, containing thymol or chloroform as biocide or no biocide, stored in sealed bottles in a coldroom at 2°C for up to 50 days (see caption to Figure 1).

Open circles: no biocide added
Open triangles: thymol added
Filled circles: chloroform added.

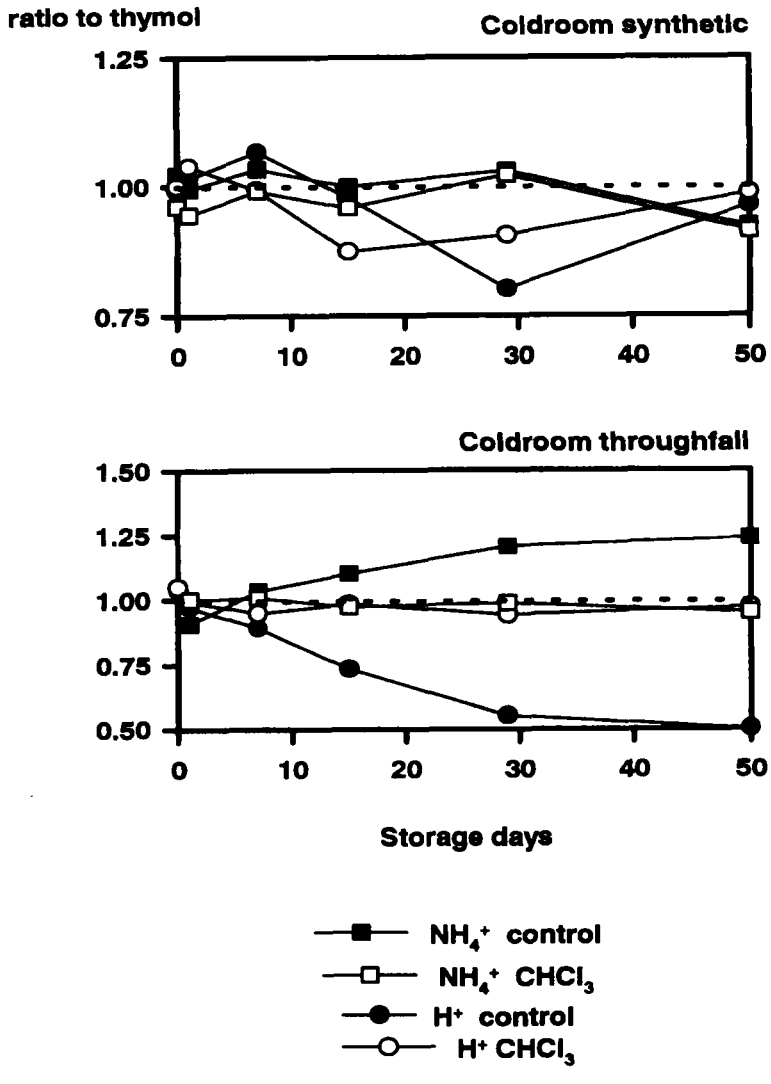

Figure 3 Concentrations of H^+, NH_4^+ ions in synthetic or real throughfall samples treated with chloroform or with no biocide ('control') relative to concentrations in thymol-treated samples stored in sealed bottles in a coldroom at 2°C for up to 50 days.

Greenhouse—unsealed samples

Changes in the composition of the synthetic samples are shown in Figure 4. A small decrease in H^+ concentration after 14 days, as seen in the coldroom, was followed by a gradual increase over the following 5 weeks. Concentrations of NH_4^+ in the untreated samples fell below those of thymol-treated samples over the 50 days, as did the NH_4^+ concentration in samples preserved with chloroform. Growth of white mould was

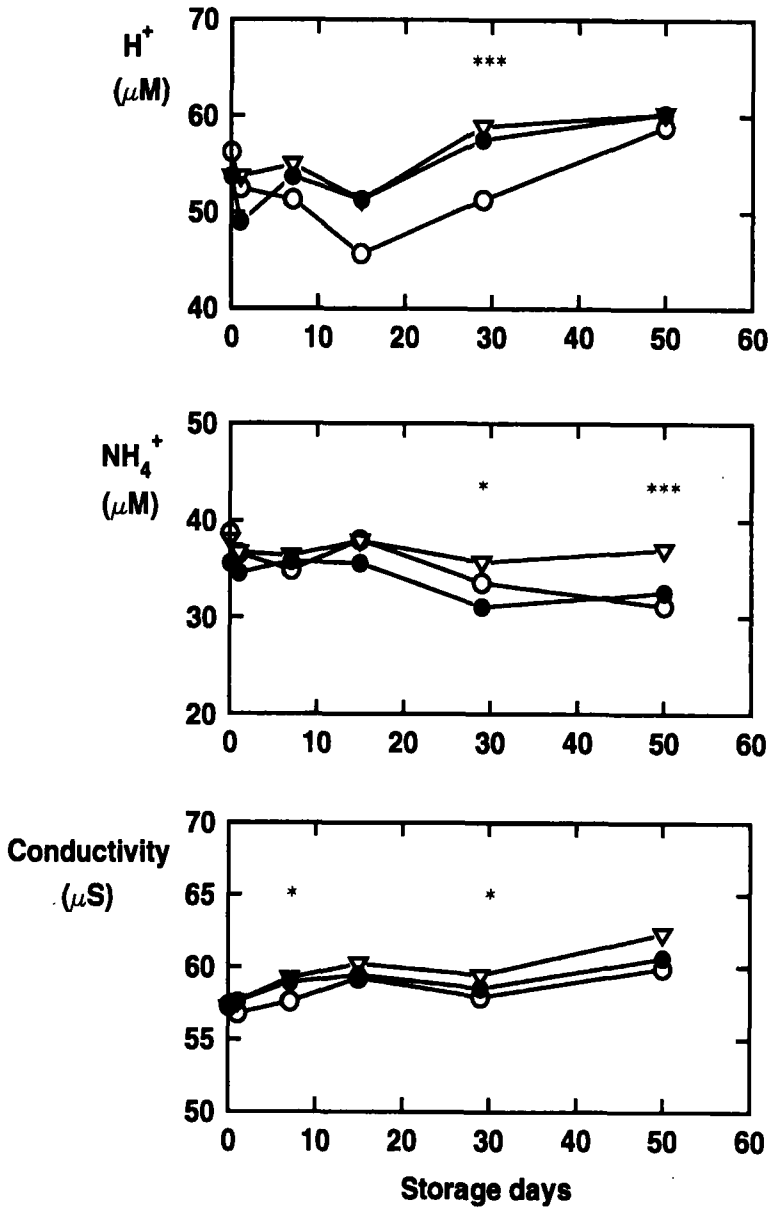

Figure 4 Concentrations of H^+, NH_4^+ and electrical conductivity of synthetic throughfall solutions, containing thymol or chloroform as biocide or no biocide, stored in unsealed bottles in a heated greenhouse at 20 (±3)°C for up to 50 days. (see caption to Figure 1).

Open circles: no biocide added
Open triangles: thymol added
Filled circles: chloroform added.

observed after 4 weeks on the samples preserved with chloroform, suggesting that the chloroform had evaporated from the unsealed bottles, and that its biocidal effectiveness had been lost. No fungal growth was observed on untreated or thymol-treated samples. Changes over time in the synthetic samples were small, however, compared with changes in throughfall samples (Figure 5). In throughfall samples there was a decrease in

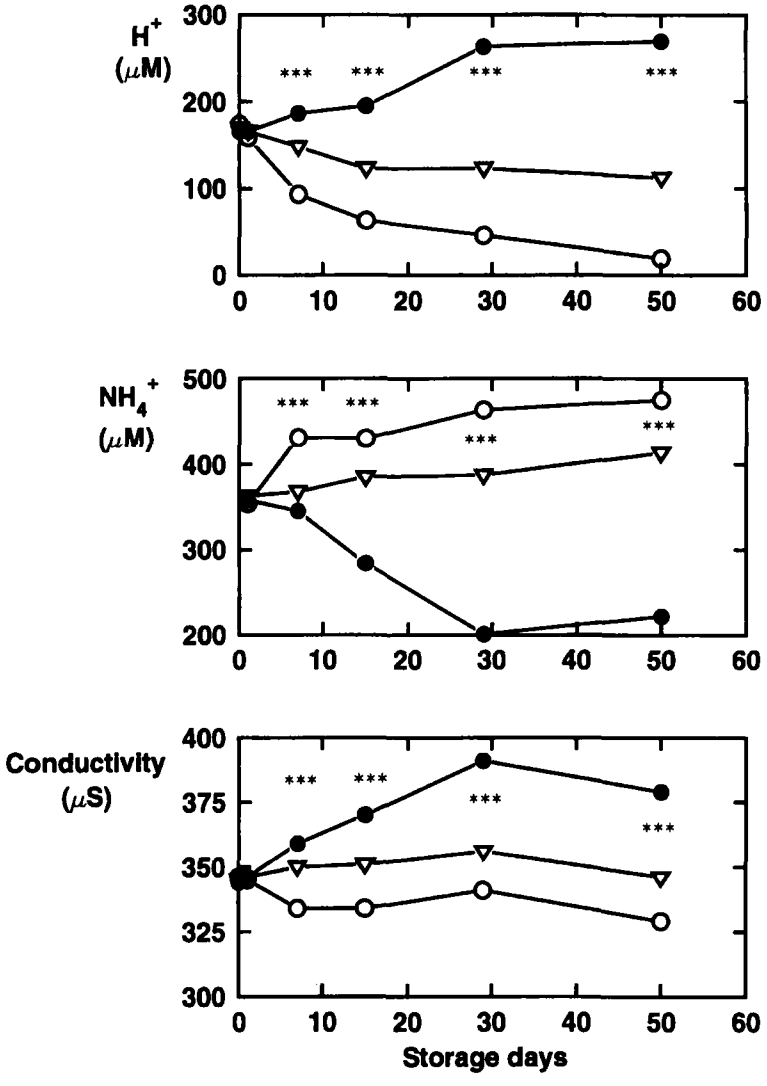

Figure 5 Concentrations of H^+, NH_4^+ and electrical conductivity of real throughfall solutions, containing thymol or chloroform as biocide or no biocide, stored in unsealed bottles in a heated greenhouse at 20 (±3)°C for up to 50 days (see caption to Figure 1).

Open circles: no biocide added
Open triangles: thymol added
Filled circles: chloroform added.

H^+ concentrations over the first 14 days in the thymol-treated samples, followed by a more gradual decrease thereafter. The chloroform-treated samples showed a marked increase in acidity, to a value twice that of the thymol-treated samples, after 4 weeks. This was matched by a decrease in NH_4^+ concentrations after 7 days, suggesting that the biocidal effects of the chloroform had been already lost. Fungal growth was also observed on these samples. In contrast, the untreated samples showed an increase in NH_4^+ concentrations, and a marked decrease in H^+, even between 1 and 7 days, followed by more gradual changes. Relative to the thymol-treated samples (Figure 6) there was no increase in NH_4^+ concentrations between 4 and 7 weeks; both sets of samples showed a slow increase in NH_4^+ concentration with time.

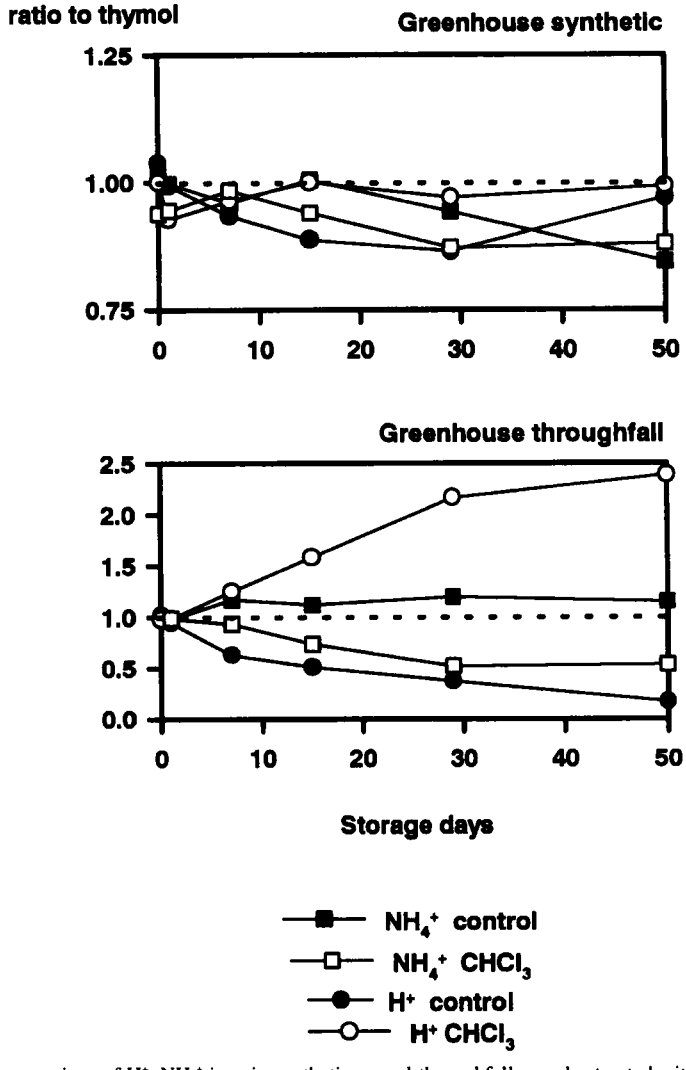

Figure 6 Concentrations of H^+, NH_4^+ ions in synthetic or real throughfall samples treated with chloroform or with no biocide ('control') relative to concentrations in thymol-treated samples stored in unsealed bottles in a heated greenhouse at 20 (±3)°C for up to 50 days.

Time trends for thymol-treated samples

Inspection of the measurements over the 50-days period (Figures 1–6) showed that samples containing thymol had the smallest variation over time. Evidence of a significant trend, possibly caused by evaporation losses (for the unsealed samples) or drift in instrument calibrations, was sought by fitting straight lines to the data for the thymol-treated samples, and testing for a significant ($p < 0.05$) slope. The results are shown in Table 2. No significant trend was expected in the sealed samples stored in the coldroom, and the only significant change detected was a very slow decrease in the NH_4^+ concentration in the throughfall samples, equivalent to 1% per week.

Evaporation from the unsealed samples in the heated greenhouse might have been expected to increase overall concentrations with time. Similarly, there was the possibility that the unsealed samples would absorb gases (e.g. NH_3, SO_2 etc.) from the atmosphere, thereby slowly changing their overall concentrations with time. In practice, the concentration of H^+ and conductivity of the synthetic samples increased by 1–2% per week, while the more acidic throughfall samples accumulated NH_4^+ at a rate of 7 μM (2%) per week, with a concomitant reduction in H^+ concentration, and no change in overall conductivity.

DISCUSSION

An ideal biocide should be non-toxic to humans, safe to handle, easy to procure and purify, should be of sufficiently low volatility that it can remain in the sample collector for periods of up to one month, should not chemically modify the rain water sample, should prevent biological consumption of labile species, and should not interfere with the subsequent chemical analysis of the ions or molecules of interest. Chloroform has been found to be effective against microbial activity, and is the biocide which traditionally has been used to preserve rain water samples from biological degradation[10,11]. Chloroform is

Table 2 Linear regressions of H^+ and NH_4^+ concentrations and conductivity of thymol-treated water samples on storage time up to 7 weeks.

Samples	*Probability that slope >< 0*	*Slope ± std.err. (μM d^{-1} or μS.cm d^{-1})*	r^2
Coldroom synthetic			
H^+	0.20	–	–
NH_4^+	0.34	–	–
Conductivity	0.33	–	–
Coldroom throughfall			
H^+	0.76	–	–
NH_4^+	0.01	−0.51 ± 0.10	0.90
Conductivity	0.30	–	–
Greenhouse synthetic			
H^+	0.03	+0.15 ± 0.05	0.68
NH_4^+	0.61	–	–
Conductivity	0.01	+0.09 ± 0.02	0.82
Greenhouse throughfall			
H^+	0.01	−1.12 ± 0.30	0.77
NH_4^+	0.001	+0.99 ± 0.12	0.95
Conductivity	0.68	–	–

usually added to the rain water after the sample is collected, to preserve the sample before chemical analysis in the laboratory, rather than as a biocide in the field during sample collection. Since the biological utilization of organic and nitrogen-containing compounds starts as soon as the rain falls, a better approach would be to add biocide to the rain water collector before it is placed in the field. Chloroform is not ideal in this respect, in that it may evaporate between site servicing visits.

Gillet and Ayers[7] reported the use of thymol as an effective biocide in formic acid solutions, and compared its efficacy with the use of chloroform. The pH of samples preserved with 1% v/v chloroform or with saturated thymol was essentially unchanged whereas the pH of untreated samples increased by at least one pH unit after 3 days.

Formic acid and acetic acids are major sources of free acidity in precipitation from remote areas of the world[12–16]. These acids rapidly disappear from samples that are not treated with a biocide, through microbial metabolism. The loss of free acidity in untreated samples between measurements at field sites and at a central laboratory, if caused solely by this process, equals the original amount of dissociated organic anions. Measurements of changes in acidity have been used to estimate amounts of dissociated organic acids in samples in which $HCOO^-$ and CH_3COO^- have not been measured directly[15].

The ionic composition of the throughfall samples was markedly different from that of the synthetic samples (Table 1). Although the concentrations of organic acids and other organic solutes were not measured in the throughfall samples, they are likely to have been large compared to rain[5]. Unpublished data (J. N. Cape) for throughfall under Scots pine (*Pinus sylvestris* L.) trees[17] showed annual average concentrations (volume weighted) of dissolved organic carbon (DOC) of 16.3 mg litre^{-1}, compared to 1.9 mg litre^{-1} in rain above the forest. The decrease in acidity for the untreated throughfall samples in the coldroom may have been caused by the biological consumption of organic acids. The ability of bacteria in aerobic environments to utilize formate and acetate as sources of carbon or energy has been well documented[18]. If bacteria had grown in the samples, they would have required a source of nitrogen[12], which was readily available as NH_4^+ or NO_3^- ions.

The concentration of H^+ in precipitation samples may also be greatly affected by the bioconsumption of NH_4^+, leading to an increase in the H^+ ion concentration. The extent of the change depends on the length of the sampling interval, the time of year and the way samples are stored prior to analysis[19]. Attempts have been made to quantify the loss (or gain) of NH_4^+ in rain water samples, depending on the type of sampler used, the duration of the collection period, and the means of storage prior to analysis[20].

In untreated samples, the bioconsumption of NH_4^+ may be the result of microbially induced oxidation (nitrification):

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$$

in the presence of nitrifying bacteria which are present in any oxygen containing environment. Alternatively, NH_4^+ may be assimilated into organic matter:

$$NH_4^+ + R.OH \rightarrow R.NH_2 + H^+ + H_2O$$

Both processes would increase the acidity of the solution, permitting the bioconsumption of NO_3^- and H^+ by pathways such as[16]:

$$NO_3^- + H^+ + ROH \rightarrow R.NH_2 + 2O_2$$

This loss of NH_4^+ and NO_3^- ions, and gain in organic nitrogen, was observed in throughfall samples to which glucose had been added to stimulate microbial activity[4].

In the untreated samples there was an *increase* in NH_4^+ concentrations in our experiment, rather than a loss of NH_4^+ as observed by Ferm[4], which was matched by a decrease in H^+ concentrations. This result suggests microbial consumption of NO_3^- ions, or of the organic nitrogen components of throughfall. The increase in NH_4^+, and decrease in H^+ concentrations, was seen in the untreated throughfall samples stored both sealed in the coldroom and unsealed in the greenhouse (compare Figures 2 and 5). Consumption of NH_4^+ was observed in chloroform-treated samples in the greenhouse, after the chloroform had evaporated. The subsequent fungal growth would account for the loss of NH_4^+ and increase in acidity. The lack of fungal growth in untreated samples suggests that the organisms responsible for NH_4^+ consumption could not compete with the NH_4^+ producing micro-organisms present in the untreated samples, but could successfully thrive when introduced to the sterilised chloroform-treated samples.

In addition to the biological assimilation of organic acids and N-containing compounds, other chemical reactions may alter the free acidity of precipitation samples between pH measurements in the field and in the laboratory. The oxidation of HSO_3^- (formed from dissolved SO_2) generates acidity[16] by conversion of the partly dissociated HSO_3^- ion to the fully dissociated SO_4^{2-} ion, leading to a decrease in pH (increase in acidity) between field and laboratory. Conversely, if particles of $CaCO_3$ or $MgCO_3$ dissolve in samples between pH measurement in the field and in the laboratory, acidity would be neutralized and pH would increase. The lack of significant change in H^+ concentrations in the real throughfall samples in the coldroom suggests that at this temperature there was little, if any, change in acidity caused by oxidation of HSO_3^- or by dissolution of alkaline particles[2,19,21].

In the experiment reported here, samples stored in sealed bottles in the coldroom and preserved with either thymol or chloroform showed no significant change over 7 weeks. Small initial changes in acidity in the synthetic samples could have been caused by absorption of acetic acid on the polyethylene walls of the bottles. Dissolution of alkaline particles, or microbial consumption of NH_4^+ are unlikely explanations of the initial changes in these samples.

CONCLUSIONS

If research objectives require an accurate measurements of the acidity of precipitation and/or complete chemical characterisation of samples, then samples must be preserved with a biocide[16]. In any studies which seek to follow the pathways of biologically labile compounds such as ammonium or nitrate ions, the likelihood of microbially induced changes during sampling or subsequent storage cannot be ignored. From our study of the biocidal effectiveness of chloroform and thymol, it may be concluded that it is necessary to preserve throughfall samples with an effective biocide if the samples are to be stored for more than 15 days, even in a cold room. Both chloroform and thymol are effective biocides for samples stored in sealed containers in a cold room for up to 50 days, but for samples which must be stored at higher temperatures unsealed, as may occur during field sampling, only thymol is an effective biocide The only disadvantage of thymol in practice, having overcome the analytical problems for ion chromatography (see above) is its toxicity. The present study clearly demonstrates, however, that saturated thymol is the biocide of choice for preventing biological degradation of throughfall samples up to the storage temperature of 20°C and storage time of 50 days.

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