N,N-Dichlorotaurine: Chemical and Bactericidal Properties

Waldemar Gottardi, Magdalena Hagleitner, Markus Nagl

Department of Hygiene, Microbiology and Social Medicine, Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Innsbruck, Austria

The biogenous antimicrobial agent *N*-chlorotaurine (NCT) converts by disproportionation to *N,N*-dichlorotaurine (NDCT) at a rate proportional to acidity. This occurs at appreciable amounts already in weakly acidic biological systems. To understand the consequences of NDCT formation, a thorough investigation of this undescribed compound was mandatory, which needed its synthesis. Differently from NCT, this was possible in the aqueous system using trichloroisocyanuric acid. While the free acid, $Cl_2HNCH_2CH_2SO_3H$, was not available in pure form, its sodium and potassium salts were analytically pure and showed melting points (decomposition) of 125−128 °C (potassium) and 162−164 °C (sodium). The sodium salt demonstrated unexpected long-term stability even at room temperature (8.4% loss of activity within 4 months). The aqueous solutions of both salts exhibited a weak acid reaction, and they were less stable than NCT. With regard to chlorination of amines (transhalogenation), NDCT was, surprisingly, less efficacious than NCT, which manifested itself by a lack of reactivity at pH < 7, for which a mechanistic explanation is given. Compared on a molar scale, NDCT was more bactericidal than NCT against the gram-negative bacteria *E. coli*, *P. aeruginosa* and *P. mirabilis*, while there was no difference concerning the gram-positive ones, *S. aureus* and *S. epidermidis*. The increase of bactericidal activity at acidic pH was the same as observed with NCT and is attributed to a higher susceptibility of bacteria in this environment. Taken together, NDCT seems not to be suited to substitute NCT as a preparation fit for medical practice.

Keywords: *N,N*-Dichloro amines; Comproportionation; Transhalogenation; Bactericidal activity

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Introduction

The highly intriguing molecule *N*-chlorotaurine (NCT) plays a fundamental role in the human immune system, and its synthesized sodium salt represents a promising antiseptic in human medicine [1−3]. Until now, its main identified functions are the bactericidal and the immune-controlling activity [4−6].

Already the first investigators of NCT discovered its inclination to disproportionate to form *N,N*-dichlorotaurine (NDCT) and taurine [7]. Because *N*-chlorotaurine and *N,N*-dichlorotaurine are strong acids, their salts are completely dissociated. The abbreviations NCT and NDCT, therefore, concern the anions $ClHN\text{-}CH_2\text{-}CH_2\text{-}SO_3^-$ and $Cl_2N\text{-}CH_2\text{-}CH_2\text{-}SO_3^-$, respectively, which are responsible for the reactions quoted in this paper. The free acid and the solid alkali salts are specified by NDCT-H and NDCT-Na, NDCT-K, respectively.

Correspondence: Waldemar Gottardi, Department of Hygiene, Microbiology and Social Medicine, Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Fritz-Pregl-Str. 3, A-6020 Innsbruck, Austria. Phone: +43 512 5073430, Fax +43 512 5072870, e-mail: waldemar.gottardi@uibk.ac.at

The rate of disproportionation (Scheme 1) increases with acidity, while the equilibrium is shifted to the right side. Thus, in 0.01 M H_2SO_4, the reaction is finished very fast (<1 min, $t_{0.5} \approx 2$ s), yielding an equimolar mixture of NDCT and taurine. At pH 5, the half-reaction time of the 1% (0.1%) solution comes to $t_{0.5} \approx 1.1$ min (≈11 min), while it is $t_{0.5} \approx 12$ min (≈120 min) at pH 7 [8]. However, these values apply to well-buffered solutions where disproportionation is impressively accelerated (see below). In fresh und unbuffered 1% (0.1%) solutions of NCT an equilibrium at pH 8.0−8.2 (7.0−7.1) is established, and disproportionation is virtually absent [8].

From these facts, it can be derived that the presence of finite concentrations of NDCT has to be considered in NCT solutions at pH <7. In biological systems, which are generally well buffered, a weakly acidic milieu is perfectly possible,

$$2\ ClHNCH_2CH_2SO_3^- + H^+ \leftrightarrow Cl_2NCH_2CH_2SO_3^- + H_3N^+CH_2CH_2SO_3^- \quad (1)$$
$$2\ NCT + H^+ \leftrightarrow NDCT + taurine$$

Scheme 1. Disproportionation of NCT.

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for example at inflammation sites [9, 10]. Therefore, also *in vivo* considerable concentrations of NDCT can be present.

Beside disproportionation, also other features of NCT are dependent on the pH, but they are not understood in all details. While the bactericidal effect of NCT increases significantly if the pH changes from 7 to 5 [5, 11], the reverse behavior is observed with the capability to chlorinate bacterial surfaces, *i.e.* the formation of durable chlorine covers [12].

To clarify these observations, a thorough investigation of NDCT was necessary, which required its synthesis. Differently from NCT, which can be isolated only as an alkali salt, namely in an alcoholic system, the free acid NDCT-H and its alkali salts, NDCT-Na and NDCT-K, could be isolated from an aqueous solution.

Results

Chemical properties

Thermal stability

The free acid and its salts exhibited unexpected thermal stability and withstood evaporation at 90 °C. The melting points of the salts were 125–128 °C (potassium) and 162–164 °C (sodium), both under decomposition.

Stability upon storage

With a loss of 8.4% within 133 days at room temperature (RT), the sodium salt unfolded a surprising stability (Figure 1), whereas the potassium salt lost 66% of its oxidation capacity at RT and 21% at 4 °C within the same period. Stored at RT, both salts produced by the time an oxidizing atmosphere (chlorine smell) in the container. This is not the case with solid NCT-Na.

Behaviour in aqueous solution

A freshly prepared 1% aqueous solution of the salts exhibited a pH ≈5.0, which soon decreased gradually to pH 4.7 after 10 min, pH 3.3 after 24 h, and pH 2.1 after 17 days at RT in the dark. During this time, also the oxidation capacity decreased, showing a distinct temperature dependency. While at RT the whole oxidation capacity had vanished after 175 days, upon storage in the refrigerator (2–4 °C) the decrease was only ~40%. No difference between the solutions of sodium and potassium salt was observed (Figure 2).

Spectra

The UV spectra of the free acid and both salts were identical and agree with the one acquired by disproportionation of NCT [8]. The IR spectrum was very similar to the one of NCT [8], except for the range of 3000–4000 cm^{-1} where the NH-band at 3260 cm^{-1} is absent and the presence of crystal water bands at 3570 and 3471 cm^{-1} can be observed (Figure 3).

Influence of buffer concentration on the formation of NDCT by disproportionation of NCT

Besides the already known impact of pH [8], also the buffer concentration had an effect on the rate of disproportionation, as shown with citrate at pH 6 and phosphate at pH 7 (Figure 4). Using 0.5 M buffer, the equilibrium was reached after ~90 min, while in 0.01 M buffer the degree of disproportionation came to less than half within the same time.

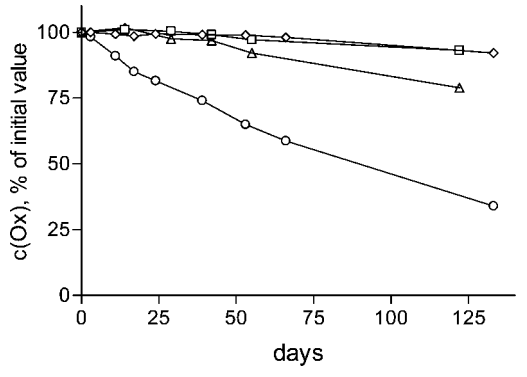


Figure 1. Stability of solid NDCT-Na and NDCT-K. At RT: (◇) NDCT-Na, (○) NDCT-K; at 2–4 °C: (□) NDCT-Na; (△) NDCT-K. Each value represents the mean ± SD of three to five replicates of iodometric titration; $p < 0.01$ between both temperatures for NDCT-K and between NDCT-K and NDCT-Na.

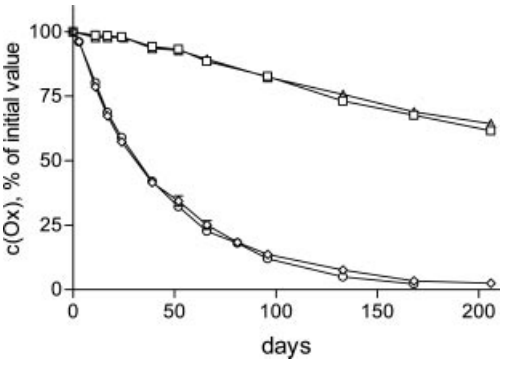


Figure 2. Stability of aqueous solutions of NDCT-Na and NDCT-K (each 55 mmol). At RT: (◇) NDCT-Na, (○) NDCT-K; at 2–4 °C: (□) NDCT-Na; (△) NDCT-K. Each value represents the mean ± SD of three to five replicates of iodometric titration; $p < 0.01$ between both temperatures, $p > 0.05$ between NDCT-K and NDCT-Na.

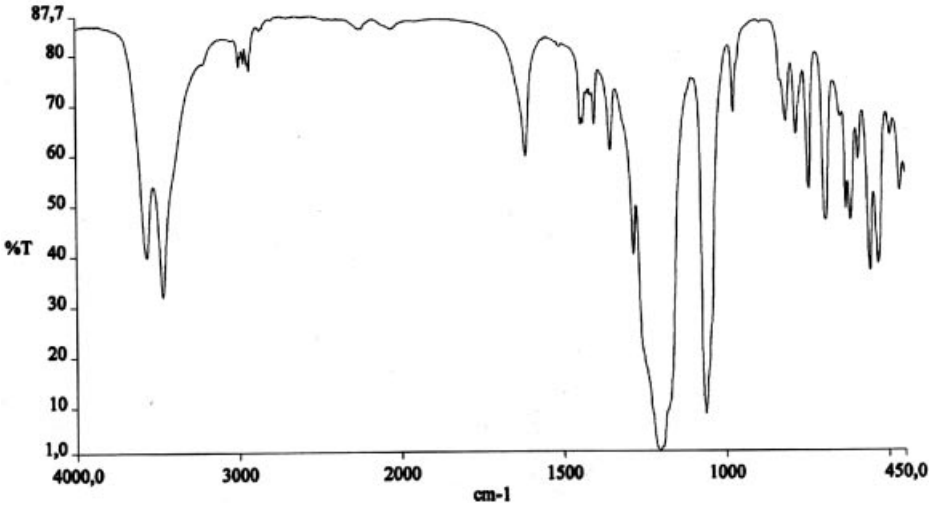


Figure 3. IR spectrum of NDCT-Na.

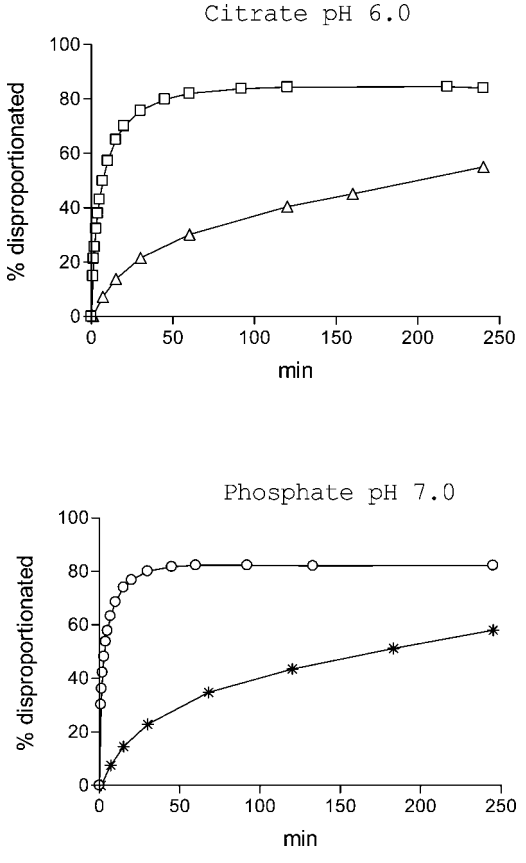


Figure 4. Rate of disproportionation of NCT (0.055 M) in citrate buffer (pH 6.0) and phosphate buffer (pH 7.0). Citrate: (△) 0.01 M, (□) 0.5 M. Phosphate: (*) 0.01 M, (○) 0.5 M. Each value represents a single UV measurement.

Absence of hydrolysis

The weakly acidic milieu (initially pH ≈ 5) that both alkali salts generate in aqueous solution points out that NDCT is not prone to undergo hydrolysis (Scheme 2). This is in contrast to NCT which produces a weakly alkaline solution [8]. The equilibrium of Scheme 2 lies therefore far on the left side.

Equilibration of NDCT with taurine (comproportionation)

The position of the comproportionation equilibrium (Scheme 3) at the final pH (which is more acidic than the initial one) expressed by the measured NCT concentration fitted well to values calculated with the reported equilibrium constants of the disproportionation of NCT [8, 13] (see Table 1).

$$Cl_2NCH_2CH_2SO_3^- + H_2O \leftrightarrow HCl_2N^+CH_2CH_2SO_3^- + OH^- \quad (2)$$
NDCT + $H_2O \leftrightarrow$ NDCTH$^+$ + OH^-

Scheme 2. Hydrolysis of NDCT.

$$Cl_2NCH_2CH_2SO_3^- + H_3N^+CH_2CH_2SO_3^- \leftrightarrow 2\ ClHNCH_2CH_2SO_3^- + H^+ \quad (3)$$
NDCT + taurine ↔ 2 NCT + H^+

Scheme 3. Equilibration of NDCT with taurine (comproportionation).

Table 1. Reaction of NDCT (0.0275 M) with taurine (0.055 M) in 0.2 M Na_2HPO_4

pH		%NCT[†]			Time[‡]	%NCT/h
Initial	Final	Exptl.	Calc.[‡‡]	Calc.[§§]	h	
7.6[§]	6.9	50.7	53.7	70.4	1.5	34
8.1[#]	7.6	71.5	71.0	83.4	2.0	36
9.2[††]	8.5	92.7	87.6	93.6	3.5	27

[†] % NCT refers to the maximally possible 0.055 M.
[‡] For attaining the equilibrium.
[§] Adjusted with H_2SO_4.
[#] pH without adjustment.
[††] Adjusted with NaOH.
[‡‡] $K_{dispr} = (4.5 \pm 0.8) \times 10^6$ [8].
[§§] $K_{dispr} = (1.07 \pm 0.15) \times 10^6$ [25].

Contrary to disproportionation in a weakly acidic milieu [8], the relative rate of comproportionation, expressed as%NCT/h, remained approximately constant within the pH range 6.9−8.5. As observed with disproportionation (Figure 4), also the rate of comproportionation increased with buffer concentration (Figure 5).

Comparison of the reaction of NDCT with different N−H compounds

Under the same conditions, *i.e.* c(R-NH_2)/c(NDCT) = 2 and 0.2 M phosphate buffer adjusted to pH 8.0, the initial rate of equilibration (Scheme 4) measured by the decrease of the concentration of NDCT was taurine >> α-alanine > glycine > β-alanine >> NH_4^+ (Figure 6). The shape of the graph of α-alanine points out that in this case an equilibrium is not approached, because of decomposition. From experiments with Chloramine T [14], it can be derived that the stability of the *N*-chloro derivatives of the investigated

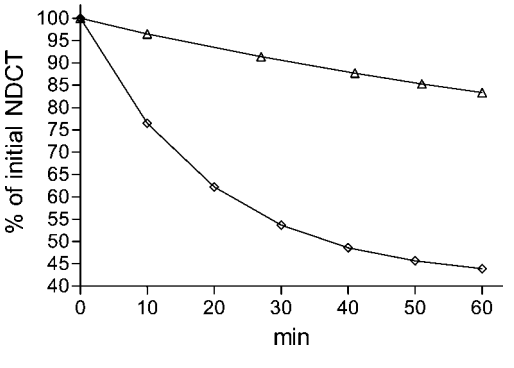


Figure 5. Rate of the reaction of NDCT (0.0275 M) with taurine (0.055 M) in 0.02 and 0.2 M phosphate buffer at pH 8: (△) 0.02 M; (◇) 0.2 M. Each value represents a single UV measurement.

$$Cl_2NCH_2CH_2SO_3^- + H_3N^+\text{-}R \leftrightarrow ClHNCH_2CH_2SO_3^- + ClHN\text{-}R + H^+ \quad (4)$$
R = H, $CH_2CO_2^-$, $CH(CH_3)CO_2^-$, $CH_2CH_2CO_2^-$, $CH_2CH_2SO_3^-$

Scheme 4. Reaction of NDCT with different N−H compounds.

amino-*carbonic* acids decreases in the order b-alanine > glycine > α-alanine. During the monitored reaction time (60 min) the pH dropped to 7.65−7.50, in case of NH_4Cl only to 7.90. This pH effect can be attributed to the transhalogenation reaction formally laid down in Scheme 4.

The same experiment conducted at pH 6.0 (0.2 M phosphate) showed not only a strong rate reduction but signifies also a shift of the equilibria of Eq. 4 (Scheme 4) towards the left side. Here, again, a clear difference between NH_4Cl and taurine is evident (Figure 6).

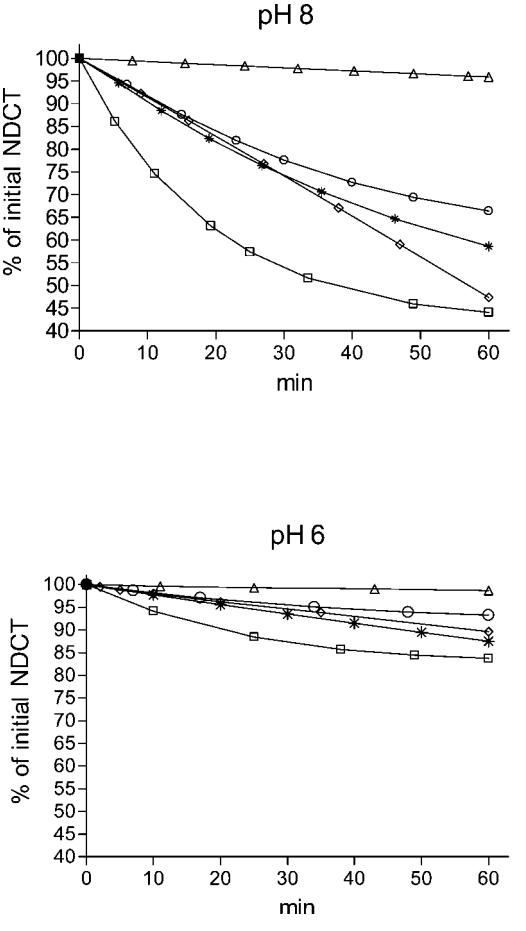


Figure 6. Rate of the reaction of NDCT (0.0275 M) with NH_4^+, β-alanine, glycine, α-alanine and taurine. (△) NH_4^+, (○) β-alanine, (*) glycine, (◇) α-alanine, and (□) taurine (each 0.055 M) in 0.2 M phosphate buffer at pH 8 and pH 6. Each value represents one single UV measurement.

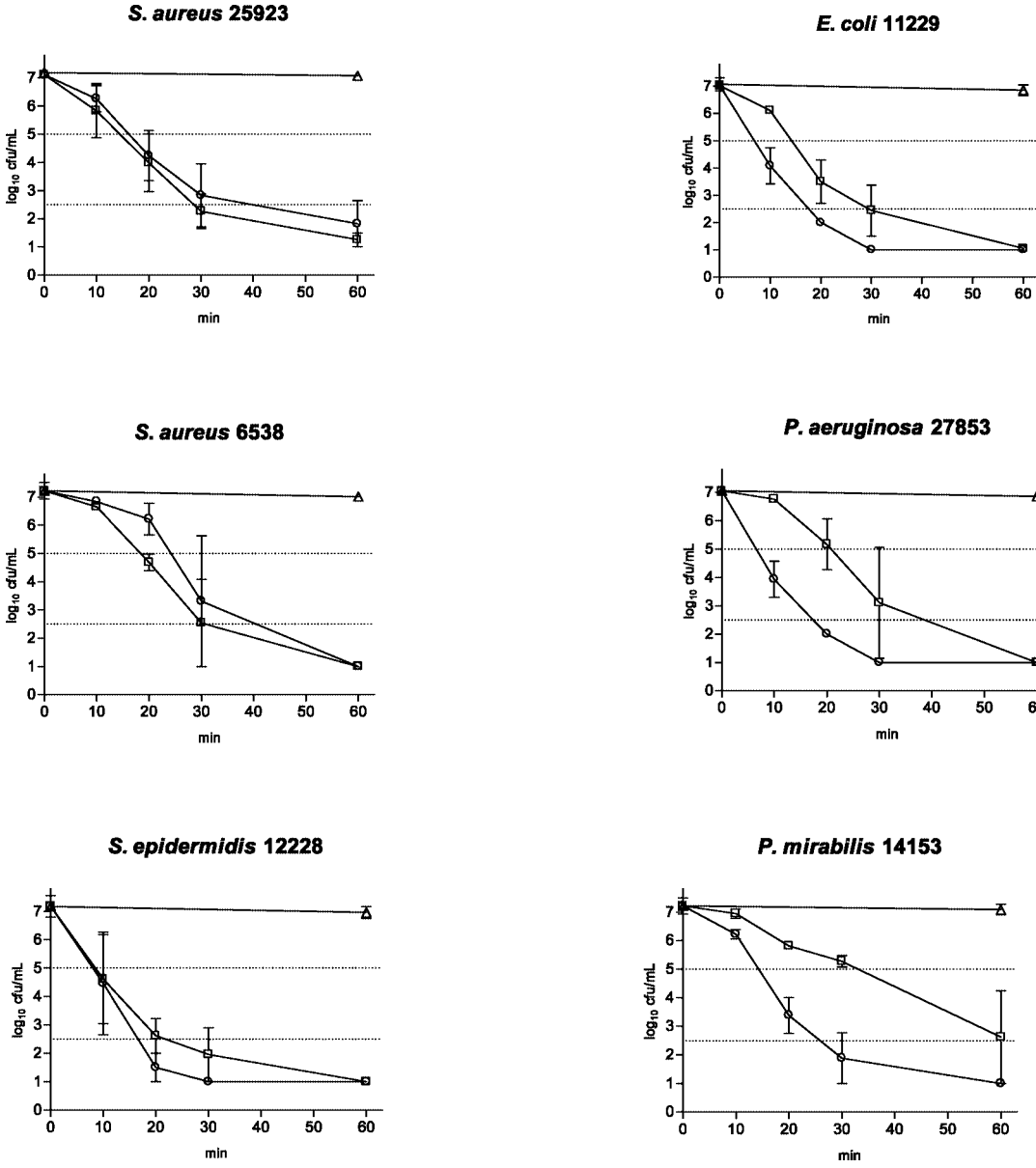


Figure 7. Bactericidal activity of each 0.055 M NCT and NDCT at pH 7. (△) Control, (□) NCT, (○) NDCT. Each value represents the mean ± SD of three to five replicates; $p < 0.01$ between NCT and NDCT for *E. coli*, *P. aruginosa*, and *P. mirabilis*; $p > 0.05$ for staphylococci.

Sulfur-containing amino acids like cysteine, N-acetyl-cysteine and methionine, however, reacted very fast with NDCT, both at pH 4.1 and pH 8.9, under complete loss of oxidation capacity.

Bactericidal activity

Since 55 mM NCT (1.0%) proved to be well tolerated by human tissue, bactericidal within minutes and therapeutically effective in previous studies [2, 11, 15–18], this concentration was chosen primarily for our experiments. The results of a comparison between 55 mM NDCT and NCT were not consistent in that the investigated gram-positive strains *S. aureus* and *S. epidermidis* were equally resistant to both compounds, while NDCT was significantly more bactericidal against the gram-negative strains *E. coli*, *P. aeruginosa* and *P. mirabilis*. This effect was the same at both pH 7 and pH 5 (Figs. 7, 8). A significant increase in the killing rate by lowering the pH from 7 to 5 is already known for NCT [5, 11]. As can be seen in Figures 7 and 8, this applied also to NDCT. For the same reduction in CFU/mL, nearly a tenfold incubation time at pH 7 was needed

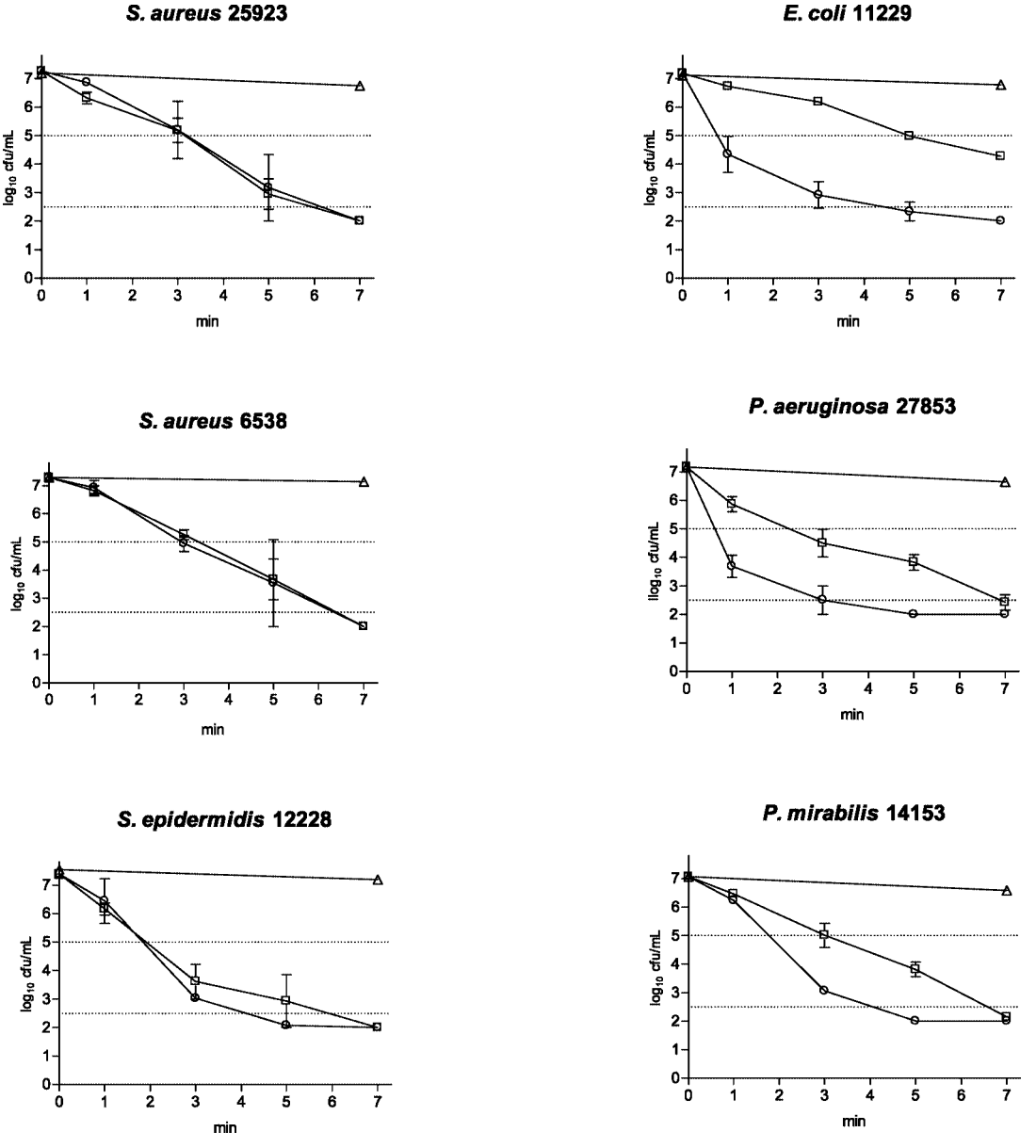


Figure 8. Bactericidal activity of each 0.055 M NCT and NDCT at pH 5. (△) Control, (□) NCT, (○) NDCT. Each value represents the mean ± SD of three to five replicates; $p < 0.01$ between NCT and NDCT for *E. coli*, *P. aruginosa*, and *P. mirabilis*; $p > 0.05$ for staphylococci.

compared to pH 5. A similar pH effect was also verified with a very low concentration of 30 μM at 37 °C (Figure 9), conditions which occur at biological sites [1, 19].

Discussion

Synthesis of NCT and NDCT

Although NCT was discovered more than 30 years ago [7], all attempts to isolate the compound from an aqueous system were unsuccessful, a failure which roots in its inclination to disproportionate to NDCT. In case of NDCT, however, disproportionation turned out to be favorable for the complete conversion into the dichloro derivative, which therefore could be easily isolated.

Stability

The gradual acidification and simultaneous loss of oxidation capacity gives evidence for a mechanism of decomposition in aqueous solution. It can be attributed to intramolecular redox and elimination reactions, for which the following scheme can be established.

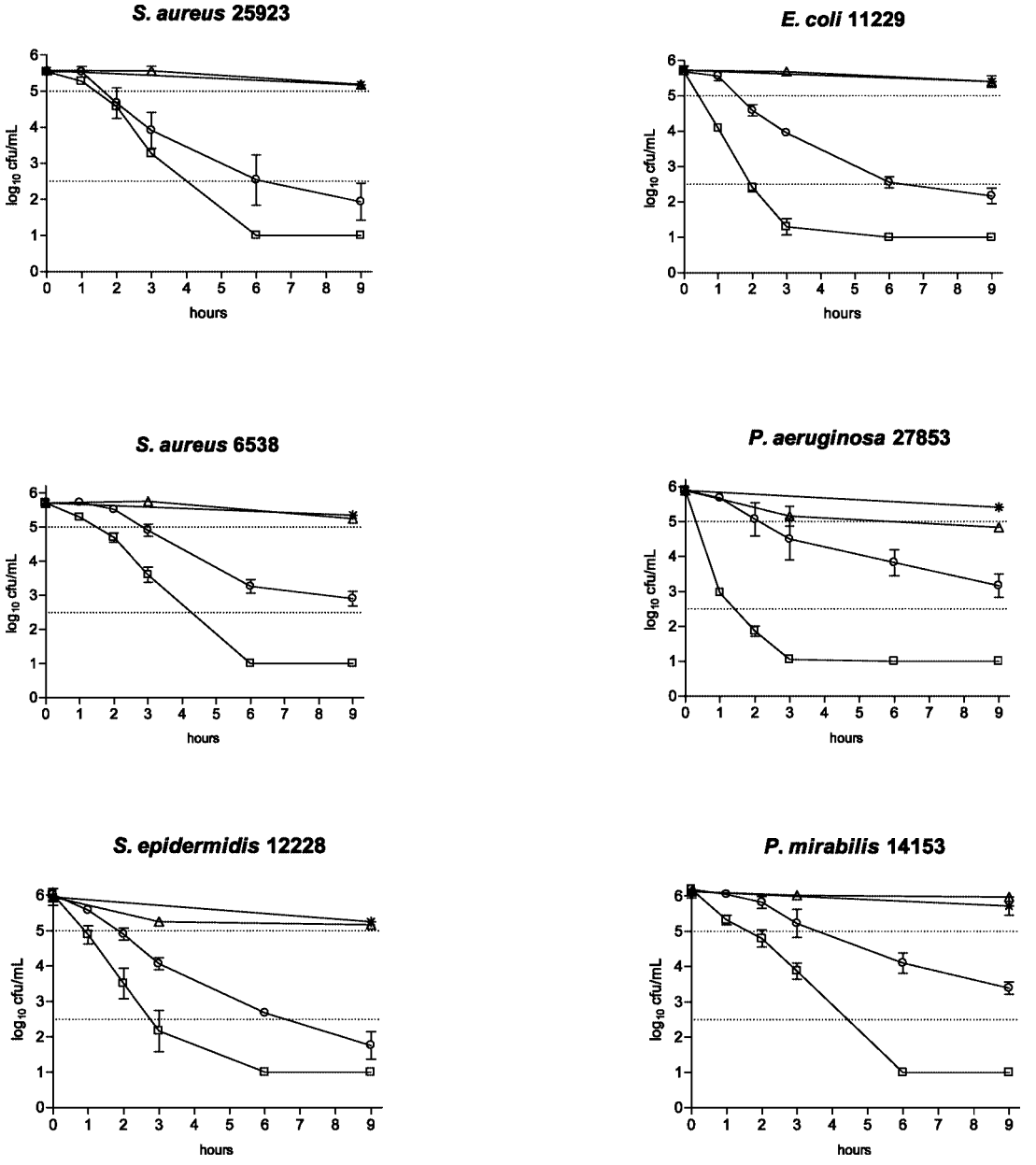


Figure 9. Bactericidal activity of 30 μM NDCT at pH 7 and pH 5. (*) Control pH 7, (△) control pH 5, (○) pH 7, (□) pH 5. Each value represents the mean ± SD of three to five replicates; $p < 0.01$ between pH 7 and pH 5 ($p < 0.05$ for *S. aureus* 25923).

As a first step, an *N*-chloroaldimine is formed (Scheme 5) which releases a second molecule of HCl or undergoes hydrolysis (Eqs. 7, 8), which finally leads to a nitril (Eq. 6) or an aldehyde (Eqs. 8, 9). Both compounds were already identified in the context of NCT decomposition [8, 20].

$$R\text{-}CH_2\text{-}NCl_2 \rightarrow R\text{-}CH{=}NCl + HCl \quad (5)$$

$$R\text{-}CH{=}NCl \rightarrow R\text{-}C{\equiv}N + HCl \quad (6)$$

$$R\text{-}CH{=}NCl + H_2O \leftrightarrow R\text{-}CH{=}NH + HOCl \quad (7)$$

$$R\text{-}CH{=}NCl + H_2O \rightarrow R\text{-}CH{=}O + NH_2Cl \quad (8)$$

$$R\text{-}CH{=}NH + H_2O \rightarrow R\text{-}CH{=}O + NH_3 \quad (9)$$

Scheme 5. Formation of *N*-chloroaldimine.

The influence of buffers on the formation of NDCT by disproportionation of NCT

Disproportionation of NCT represents a specific form of chlorination, namely an auto-chlorination. The results presented here (Figure 4) uncover, in addition to the influence of pH, a major effect of the buffer concentration upon the transhalogenation rate. They comply with kinetic studies

$$R\text{-}NHCl + H_2PO_4^- \leftrightarrow R\text{-}NH_2Cl^+ + HPO_4^{2-} \quad (10)$$

Scheme 6. Formation of the actual reactive species $NCTH^+$.

$$R\text{-}NH_3^+ + HPO_4^{2-} \leftrightarrow R\text{-}NH_2 + H_2PO_4^- \quad (11)$$

Scheme 7. Equilibration of buffer with the amine.

gained in the presence of methoxyacetic acid/methoxyacetate buffers [13]. A similar boosting effect has been observed in a study on bacterial chlorine covers [12] and was explained by the proton-donating properties of buffers which favor the formation of the actual reactive species $NCTH^+$ even at pH 7 [8] according to Scheme 6.

However, also an equilibration of buffer with the amine can be considered, which would increase the concentration of the non-protonated amine representing the actual nucleophilic agent (Scheme 7).

Figure 5 discloses that also in the case of the inverse reaction, *i.e.* comproportionation, buffer concentration has a crucial influence on the reaction rate. Since formation of the protonated species ($R\text{-}Cl_2H^+$, see below) is less probable, a substantial contribution of the equilibrium (Eq. 11) seems plausible.

General readiness for transhalogenation of NCT and NDCT

The term transhalogenation was defined for the transfer (exchange) of positive halogen between amine compounds. Contrary to the substitution of a C-H bond, it occurs rather fast already at RT and needs no catalyst, and it is not connected with a loss of oxidation capacity [8]. The differing behavior of NCT and NDCT can best be exposed by the relation between disproportionation (Scheme 1) and comproportionation (Scheme 3). Both imply a transhalogenation reaction whose equilibrium is highly pH dependent. A simplified approach could be: disproportionation of NCT occurs readily at pH < 7, and comproportionation of NDCT and taurine at pH > 7.

Our results demonstrate that this concept is not limited to the equilibria between taurine, NCT, and NDCT, but apply generally to equilibria between unsubstituted amines, mono-chloro amines, and/or dichloro amines (Scheme 8):

$$R\text{-}NHCl + R'\text{-}NH_2 \leftrightarrow R\text{-}NH_2 + R'\text{-}NHCl$$
$$R\text{-}NHCl + R'\text{-}NHCl \leftrightarrow R\text{-}NH_2 + R'\text{-}NCl_2$$

Scheme 8. Equilibration between unsubstituted amines, mono-chloro amines, and/or dichloro amines.

Anyway, the readiness of NDCT for transhalogenation is actually given at pH > 7. The confirmation of a reaction of NDCT with taurine at pH 7.1 needed a 400-fold surplus of taurine, at least [13]. However, in the presence of a substantial buffer concentration, the reaction could be proved even at pH 6 (Figure 6).

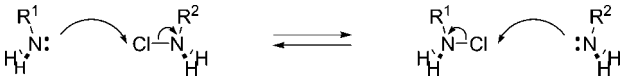
Reactivity of NDCT against defined NH compounds

The observed rates at pH 8 (Figure 6) reveal the order taurine >> α-alanine > glycine > β-alanine >> NH_4^+, which is rather bewildering. Since the reaction shown in Eq. 4 (Scheme 4) proceeds *via* a nucleophilic substitution (see scheme below), the unprotonated amines act as nucleophilic agents, and their pK_a values should provide an indication of their relative concentration at a given pH. However, the pK_a values of 9.19, 9.246, 9.91, 10.01 and 10.24 for taurine, NH_4^+, glycine, α-alanine, and β-alanine, respectively [21], disclose that this concept does not coincide with the reaction rates, most notably of taurine and NH_4^+ (Figure 6). The strikingly low reactivity of NH_4^+ can be ascribed to the absence of an inductive effect. In case of attached alkyl groups, the C-H functions act as electron donors and increase the nucleophilicity of the amine function. However, this approach conflicts to a certain degree with the rates of glycine, α- and β-alanine.

The lower reactivity at pH 6, on the other hand, can easily be explained with the actual concentration of the non-protonated amine compounds, which is significantly lower than at pH 8. From the pK_a values it can be derived that, *e.g.* for taurine (β-alanine), the concentration of the free amine form is by a factor of 94.0 (99.5) higher at pH 8 compared to pH 6.

General reactivity of NDCT compared to other active chlorine compounds

The ability to produce chlorine covers on protein matrices allowed the ranking of active chlorine compounds according to their chlorinating potency regarding transhalogenation. In case of chlorine covers on human skin, the order $HOCl/OCl^-$ > Chloramine T > NH_2Cl > NCT was found [22], and in case of bacterial covers, it was DCI-Na > Chloramine T > NCT > NDCT [12]. Combining both rankings evidences the very last position of NDCT. This is rather surprising because it possesses two chlorine atoms on the same nitrogen, suggesting a higher reactivity in terms of transfer of positive chlorine. The lesser potency of NDCT [12] can be attributed to mechanistic effects. As pointed out in [8], the halogen transfer between NCT and an amine compound implies an attack of the free electron pair of the amino group of the acceptor molecule $R_1\text{-}NH_2$ towards the chlorine atom of the protonated donor molecule $R_2\text{-}NH_2Cl^+$ according to a nucleophilic substitution:



Scheme 9. Halogen transfer between NCT and an amine compound.

In case of NDCT, it seems plausible that two chlorine atoms at the same nitrogen cause a positive partial charge high enough to impede the intermediary formation of the protonated donor molecule $R\text{-}NCl_2H^+$. This could explain why NDCT, contrary to NCT, is not suited in such a rate for a chlorine transfer and why comparatively weak chlorine covers are formed [12]. For the same reason, also hydrolysis according to Eq. 2 (Scheme 2) does not take place.

In spite of these considerations a transhalogenation is conceivable also with the non-protonated NDCT molecule, where the second chlorine atom induces a positive partial charge at the chlorine reacting with the nucleophilic agent. However, this partial charge is less than at the protonated NCT molecule which bears a real charge. NDCT, therefore, reacts markedly only in a weakly alkaline milieu where the portion of the free amines is high. Among the *N*-chloro species of taurine the following order in terms of electrophilicity seems plausible: $R\text{-}NClH_2^+ > R\text{-}NCl_2 > R\text{-}NHCl$ resp. $NCTH^+ > NDCT > NCT$.

Bactericidal activity

Regarding the experiments conducted with the same molar concentration of 55 mM (Figs. 7, 8), one would expect a stronger activity of NDCT because its oxidation capacity is twice as much as that of NCT. This was in fact observed both at pH 5 and 7; however, only with gram-negative bacteria. The lower sensitivity of gram-positive bacteria to NDCT cannot be explained until now. The throughout higher bactericidal activity of NDCT at pH 5 (Figs. 6, 7) complies with the behavior of NCT [5, 11], but also with other active chlorine compounds like hypochlorite [23] and Chloramine T [24]. For the latter compounds, a pH shift from 7 to 5 implies a substantial change in the equilibrium concentrations of the active species: in hypochlorite solutions, *e.g.*, an increase of the ratio $[HOCl]/[OCl^-]$ from 3.4 to 344. Accordingly, the higher bactericidal effect of hypochlorite at pH 5 was explained [23]. Since the molecule NDCT does not undergo any change this approach does not work. An influence of pH upon the susceptibility of bacteria towards chlorinating agents is conceivable. This applies also to NCT whose increase of killing activity – based on the results presented here – cannot be explained by disproportionation.

A possible explanation could rely on a change of electric charges that occurs if the pH changes from 7 to 5. Since bacterial membranes are bearing negative charges, the approach of the anions NCT and NDCT is hampered in a neutral environment. If the solution becomes acidified, an increase of non-ionized molecules and a decrease of negative charges on the bacterial surface take place, resulting in a better access of the chlorinating agents. Concerning the mechanism of bacterial kill, it can be stated that transhalogenation is not the main cause. If this was true, NDCT with its significantly minor potency to form chlorine covers [12] would be less active than NCT, which was not observed. The main cause for bacterial kill might be found in the oxidation of thiols which occurs in the whole pH range with a high rate and which causes irreversible mutations in the protein structure.

The consequence of NDCT formation by disproportionation at biological sites

Three features can be specified:

(1) Based on the fact that two molecules of NCT yield one of NDCT, our results suggest rather a decrease of bactericidal activity of NCT caused by disproportionation, at least in the case of gram-positive bacteria.

(2) Because NDCT produces weaker chlorine covers on bacteria than NCT [12], disproportionation might also induce a weakening of the post-antibiotic effect, which is beneficial in overcoming infections by the immune system [5, 15].

(3) Another drawback might originate in the minor stability of NDCT in solution (Figure 2), as NDCT, in contrast to NCT, decomposes under acidification. This favors the rate of disproportionation even more.

In summary, it can be postulated that appreciable concentrations of NDCT will usually be unfavorable for the performance of NCT. That is why conditions that promote disproportionation, *e.g.* $pH < 7$, should be avoided when applying NCT as an antiseptic in human medicine. For the same reasons, NDCT-Na, despite of its surprising stability, seems not suitable as a substitute for NCT-Na.

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Experimental

Chemical experiments

Reagents

Trichloroisocyanuric acid (TCI) was purchased from Fluka AG and contained 44.3% Cl^+ (assayed iodometrically), which corresponds to M_r(eff) = 240.0. All other chemicals were from Merck and of the highest available purity.

Analytic methods

Oxidation capacity was assayed by iodometric titration keeping the sequence acidification (50% acetic acid) of the analyte solution *before* addition of potassium iodide. The reverse order resulted in a value ~30% too low for NDCT, while it had no influence on the assessment of NCT. The origin of this effect is not quite clear. Since the reaction of an *N*-chloro compound with iodide is connected with an initial strong alkalization (>N-Cl + $2I^-$ + H_2O → >N-H + I_2 + OH^-) an intramolecular redox reaction is conceivable (see Scheme 5, Eqs. 5, 6) that is connected with a loss of oxidation capacity.

Preparation of an aqueous solution of NDCT-H

To a stirred ice-cooled solution of 1.25 g taurine (0.01 mol) in 100 mL water was gradually added 1.6 g trichloroisocyanuric acid (0.02 mol Cl^+). After 0.5 h of stirring at 1−2 °C, the precipitated cyanuric acid was removed by filtration. On the basis of the UV spectrum ($A_{302.4}$ = 0.360, $A_{251.5}$ = 0.254) and the known molar absorption coefficients [8], the resulting strongly acidic solution contained 0.109 M NDCT-H. Iodometric titration yielded 0.207 ± 0.002 M Cl^+, which also indicates a quantitative reaction.

Isolation of free acid

The aqueous solution containing NDCT-H (see above) was vacuum-evaporated (water-jet vacuum pump), whereby the temperature of the water bath was increased to 90 °C at the end. The resulting pungently smelling, micro-crystalline material-containing oil was suspended in 15 mL 1,2-dichloroethane and once more vacuum-evaporated after filtration. The remaining hygroscopic clear yellowish oil (1.7 g; calculated 1.94 g) was free of cyanuric acid and represented for the most part the free acid NDCT-H. By iodometric titration, we found 32.4 ± 0.3% Cl^+ (calculated 36.54% Cl^+). The UV spectrum showed the NCl_2 band at 302.4 nm. It was neither possible to purify (*i.e.* to remove water) the very aggressive, *i.e.* strongly acidic and highly oxidizing, substance nor to record an IR spectrum.

General procedure for preparing alkali salts by neutralization of NDCT-H

To the ice-cooled aqueous solution of NDCT-H was added the calculated amount of NaOH or KOH up to pH ≈ 5. The resulting solution was then vacuum-evaporated as described above. The crystalline residue containing the alkali salt was purified by re-crystallization, resulting in 1 g of the potassium salt from 13 mL boiling methanol and 1 g of the sodium salt from 15 mL boiling ethanol/methanol (4:1). NDCT-Na: found 31.77 ± 0.30% Cl^+ (N = 3); calculated: 32.82% Cl^+ (semi-hydrate: 31.51% Cl^+). The sodium salt contained crystal water (bands at 3565 and 3470 cm^{-1} in the IR spectrum) which disappeared in the vacuum over P_2O_5. NDCT-K: found 30.00 ± 0.072% Cl^+ (N = 3); calculated: 30.55% Cl^+.

Monitoring the reactions of NDCT

Equilibria between NCT, NDCT and taurine (disproportionation and comproportionation) were monitored photometrically using the absorption maxima of NCT (λ_{max} = 250.9 nm, ε = 397.4 L mol^{-1} cm^{-1}) and NDCT (λ_{max} = 302.4 nm, ε = 332.9 L mol^{-1} cm^{-1}) as set forth in [8]. In case of reactions with NH_4^+/NH_3 (ammonium chloride), glycine, α- and β-alanine, the absorption at 302.4 nm was used, which allowed to assess the temporal decrease of NDCT.

Microbiological experiments

Bacteria

Bacterial strains (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Proteus mirabilis* ATCC 14153, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 27853 deep-frozen for storage were grown overnight on tryptic soy agar (Merck, Darmstadt, Germany). Colonies from this agar were grown in tryptic soy broth (Merck, Darmstadt, Germany) at 37 °C overnight and twice washed with saline.

Killing tests

Bacteria were diluted in buffered NDCT (NCT) solution to $0.6 \times 10^7 - 3.5 \times 10^7$ CFU/mL. Immediately and subsequent to different incubation times at RT, aliquots were removed and NDCT (NCT) was inactivated with sodium thiosulfate. Undiluted aliquots (50 μL) as well as 100-fold dilutions in saline (50 μL) were spread onto tryptic soy agar plates with an automatic spiral plater (Don Whitley Scientific Limited, West Yorkshire, UK) in duplicates, allowing a detection limit of 10 CFU/mL. Plates were grown at 37 °C, and CFU were counted after 24 and 48 h. Controls without NCT were treated in the same way.

Statistics

One-way analysis of variance (ANOVA) and Dunnett's Multiple Comparison test (GraphPad Software Inc., CA, USA) was applied; *p* values <0.05 were considered significant.

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