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Factors Influencing the Effectiveness of Swimming Pool Bactericides

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Techniques for culturing, harvesting, and testing bacteria to evaluate bactericidal chemicals for swimming pools are described. Concentrations of 25, 50, and 100 mg of the chlorine stabilizer cyanuric acid per liter increased the time required for a 99% kill of *Streptococcus faecalis* by 0.5 mg of chlorine per liter at *p*H 7.4 and 20 C from less than 0.25 min without cyanuric acid to 4, 6, and 12 min, respectively. The effect of concentrations of ammonia nitrogen in the range found in swimming pools on the rate of kill of 0.5 mg of chlorine per liter and of chlorine plus cyanuric acid was tested. At concentrations of ammonia nitrogen greater than 0.05 mg per liter, faster rates of kill of *S. faecalis* were obtained with 100 mg of cyanuric acid per liter plus 0.5 mg of chlorine per liter than with 0.5 mg of chlorine per liter alone. When water samples from four swimming pools with low ammonia levels were used as test media, 0.5 mg of added chlorine per liter killed 99.9% of the added *S. faecalis* in less than 2 min, but water from a pool with a large number of children required 60 to 180 min of treatment.

In the present study of the compatability of chemicals occurring in swimming pools with the bactericidal properties of chlorine, there appeared to be a need to demonstrate some of the factors that affect the bactericidal properties of chlorine in laboratory evaluations. The importance of the methods of harvesting and handling the bacteria and the media used for such tests is emphasized by the very different results from similar experiments reported by various authors (4, 9, 13; J. R. Andersen, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1963). In this paper, the effect of the chlorine stabilizer cyanuric acid on the rate of kill by different chlorine concentrations is demonstrated as an example of how reproducible laboratory techniques can be used for evaluations of swimming pool additives under conditions of constant *p*H and other environmental factors. In addition, the effect of ammonia nitrogen in the concentration range reported for swimming pools (2, 3, 7) on the rate of kill by chlorine and chlorine plus cyanuric acid is reported. The value of using the rate of kill obtained with free, available chlorine as the basis for acceptance of bactericides for swimming pool use (10) is discussed in view of the effect ammonia nitrogen, at levels found in swimming pools, has on the rate of kill by chlorine.

MATERIALS AND METHODS

All glassware used was acid-washed. Erlenmeyer flasks used for tests were chlorinated (approximately 10 mg per liter) overnight or longer, rinsed with distilled water, and sterilized with hot air. All solutions used in tests were made with sterile water. Agar for slants and plates was dehydrated Brain Heart Infusion Agar (Difco) reconstituted according to the manufacturer's directions. Buffer solution was made by dissolving 34 g of KH_2PO_4 in 1,000 ml of double-distilled water, and adjusting the *p*H to 7.4. Stock chlorine solution was made by diluting 4 ml of a commercial 5.25% sodium hydrochlorite solution to 100 ml with distilled water, and was stored in a refrigerator. The chlorine-free, chlorine demand-free, buffered water used for dilutions, harvest, and media was prepared as follows. Double-distilled water was chlorinated to about 2 to 4 ppm and stored for at least 48 hr. A 5,225-ml amount of the chlorinated water was buffered with 275 ml of stock solution at *p*H 7.4. This solution was dechlorinated with a dilute sodium sulfite solution, brought to a full boil, cooled to 20 C overnight, and used for tests.

Chlorine was determined according to the orthotolidine-arsenite method (1). The 5-min color by orthotolidine (free, available plus combined, available chlorine) was measured after arsenite was added. Absorption was measured in a Klett-Summerson colorimeter equipped with a blue filter (1.0 mg per liter gave a reading of 385).

A sterile sodium sulfite solution (200 mg per liter) was used as a neutralizer for chlorine. Portions (5-ml) of sodium sulfite solution in screw-capped test tubes were autoclaved at 120 C for 15 min. The neutralizer did not have any harmful effects on survival of organisms. Neutralizer was initially used in tests determining the kill rate in chlorine and in chlorine plus cyanuric acid (5 ml of samples plus 5 ml of neutralizer). Later samples were neutralized by adding 1 ml of test water to 9 ml of neutralizer before plating.

Streptococcus faecalis (ATCC-8043) from Wisconsin Alumni Research Foundation was used as the test organism. The stock culture was transferred monthly, incubated for 6 to 8 hr at 37 C, and stored in a refrigerator. Beginning with the stock culture, daily transfers were made for about 30 days and were incubated at 37 C for 20 to 24 hr. To harvest *S. faecalis*, 16-oz prescription bottles were used with 30 ml of agar solidified in a horizontal position. The growth of a daily slant was washed off with 8 to 10 ml of test water and distributed over the agar surface in the bottle; the excess was drained off into chlorinated water. Inoculated bottles were incubated for 24 hr at 37 C. The cells were harvested by washing 30 ml of test water over the agar surface and filtering this suspension through Whatman no. 2 filter paper with a slight vacuum. The filtered suspension was centrifuged at 2,000 $\times$ *g* for 15 min. The supernatant fluid was discarded, and the bacteria were resuspended in test water. From this stock solution, a standard suspension was made with an absorption of 0.18 to 0.19 in 1-inch (2.54-cm) tubes. A 1-ml amount of the standard suspension was used in 100 ml of test medium and gave a concentration of 900,000 to 1,100,000 cells per ml of medium.

Test procedure. Test media were prepared in chlorine-soaked, sterile 250-ml Erlenmeyer flasks. At zero-time, 1 ml of standard *S. faecalis* suspension was added to 99 ml of medium composed of test water and compounds to be tested. At various times, samples were taken, neutralized if necessary, diluted, and added to tubes with molten agar. The contents were plated in a sterile petri dish. The surviving number of *S. faecalis* cells were counted after 48 hr of incubation at 37 C.

RESULTS

Source of bacteria. To obtain reproducible numbers of bacteria per milliliter of test medium, the system of culture and harvesting described was used. The use of optical-density measurements to adjust the concentration of bacteria in the washed harvest suspension so that predictable concentrations of bacteria could be obtained has been found to be satisfactory (12; Andersen, Ph.D. Thesis). A direct relationship exists between the numbers of bacteria, in the range of 50 $\times$ 10^6 to 500 $\times$ 10^6 per ml, and the optical densities of the suspensions when measured in 1-inch tubes at a wavelength of 600 mμ.

By use of the above technique, it was possible to obtain a stock concentration of about 100,000,000 cells per ml, which when diluted with 99 ml of test water produced a final concentration of 1,000,000 cells per ml. The results of 20 tests showed that a variation could be expected of $\pm 3\%$ in the number of bacteria per control plate (usually 90 per plate) in five plates (3 at zero-time and 2 at final time) of any single experiment, and $\pm 4\%$ from experiment to experiment.

Effect of washing bacteria. A comparison of the percentage of bacteria surviving in different chlorine concentrations after a 1-min treatment time was carried out with bacteria with and without washing. The bacteria used were obtained either directly after rinsing off the stock agar surface and filtering, or from the filtrate which had been centrifuged and resuspended in chlorine demand-free water. Figure 1 presents averages of data obtained in four such experiments. The solid line emphasizes the results obtained with unwashed bacterial suspensions and the dashed line emphasizes results with washed suspensions.

In general, in test media containing approximately 1,000,000 bacteria per ml, there was a decrease in the concentration of chlorine required to obtain a 99% kill within 1 min from approximately 0.3 mg of chlorine per liter for unwashed suspensions to 0.1 mg of chlorine per liter for washed suspensions. Related tests were made comparing chlorine demand of wash water from uninoculated media bottles with that of unwashed bacterial suspensions diluted to give a concentration of approximately 1,000,000 bacteria per ml in the final test medium. An equivalent dilution in the case of wash water from uninoculated media bottles was also tested. Experiments were carried out in test water with initial chlorine concentrations of 0.2 to 2 mg per liter. The averages of results of three experiments at initial chlorine concentrations of 0.4 to 0.5 mg per liter are presented in Fig. 2 as the chlorine

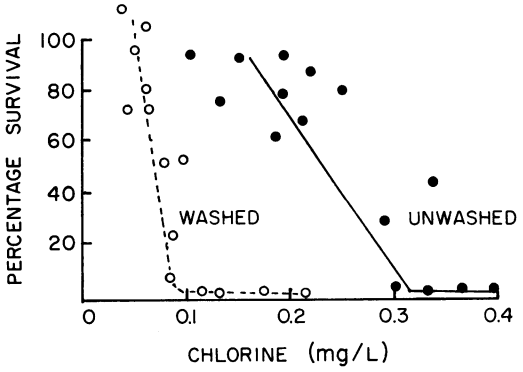

FIG. 1. *Comparison of washed and unwashed suspensions of Streptococcus faecalis as the percentage surviving after 1 min in different chlorine concentrations.*

demand (loss from the initial concentration) versus time in minutes.

The results presented in Fig. 2 were typical of those from all other concentrations of chlorine tested. In general, the chlorine demand of bacterial suspensions was approximately 75 to 85% due to the medium carried over with the bacteria and 15 to 25% due to the bacteria. The necessity of using washed bacteria to obtain reproducible kill rates with low concentrations of free available chlorine is evident.

Chlorine demand versus number of bacteria. A series of eight tests was carried out to measure the chlorine demand of different concentrations of washed *S. faecalis* cells when suspended in a relatively chlorine demand-free medium. Data are presented in Fig. 3 from a typical experiment in which the initial chlorine concentration was 0.25 mg per liter, and the loss of chlorine in suspensions of 0.25, 0.5, 1, 2, and 4 $\times$ 10^6 bacteria per ml was followed for 30 min.

These tests indicate that increasing the number of bacteria in a test with chlorine will increase the chlorine demand of the system. The actual amount of chlorine consumed by a concentration of 10^6 cells per ml can be estimated to amount to approximately only 0.01 mg per liter per min. It should be noted that the chlorine demand of bacterial suspensions continues after the bacteria have been killed (less than 1 min at a chlorine concentration of 0.25 mg per liter).

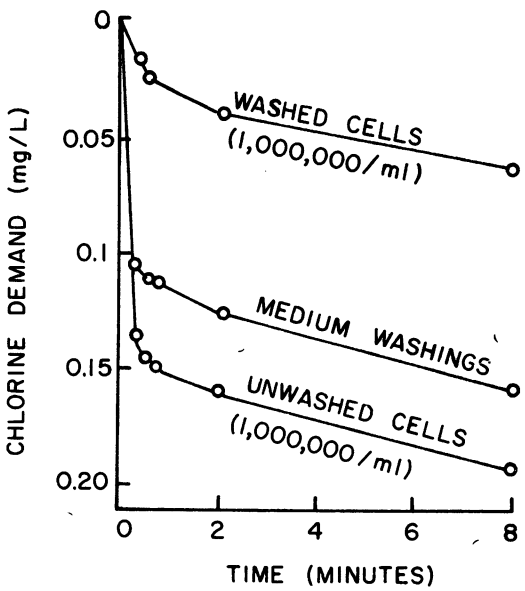

FIG. 2. *Chlorine demand of suspensions of Streptococcus faecalis and washings from uninoculated medium (Brain Heart Infusion Agar) diluted similarly as bacterial suspensions.*

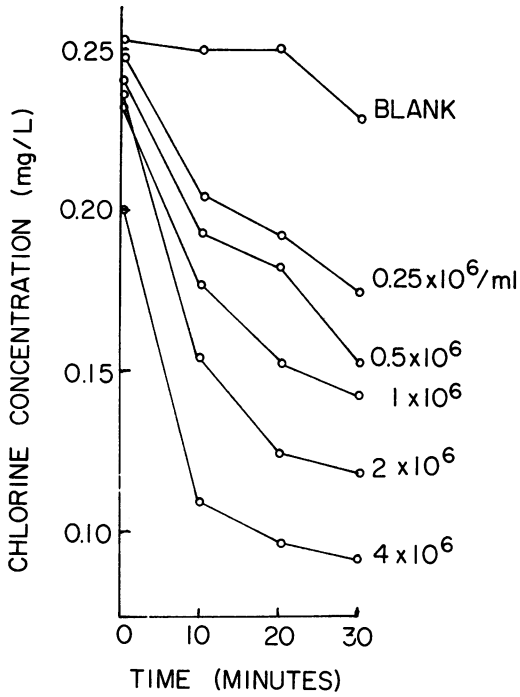

FIG. 3. *Chlorine consumed by different concentrations of washed cells of Streptococcus faecalis.*

Cyanuric acid versus rate of kill. The effects 25, 50, and 100 mg of cyanuric acid per ml on the rate of kill of bacteria by nominal concentrations of chlorine at *p*H values near 7 have been well established by Andersen (Ph.D. Thesis) and Stuart and Ortenzio (13). Averages of results obtained under the conditions described earlier in the present investigation are presented in Fig. 4 as the time required for a 99% reduction in numbers of bacteria from the initial concentration of 1,000,000 per ml.

It is evident from these data that cyanuric acid at concentrations of 25, 50, and 100 mg per liter reduces the bactericidal action of chlorine and that this effect increases with increasing amounts of cyanuric acid. The time required for 99% of the bacteria to be killed in the presence of 0.5 mg of chlorine per liter was 0.25 min or less with chlorine alone and approximately 4, 5, and 12 min with 0.5 mg of chlorine per liter plus 25, 50, and 100 mg of cyanuric acid per liter, respectively.

Studies on the loss of chlorine from test waters (chlorine demand) indicated that, during the first 5 hr after chlorination, the presence of cyanuric acid concentrations of 25, 50, and 100 mg per liter had little effect on the loss of chlorine from solution.

Effect of ammonia and urea. That ammonia will affect the bactericidal properties of chlorine has

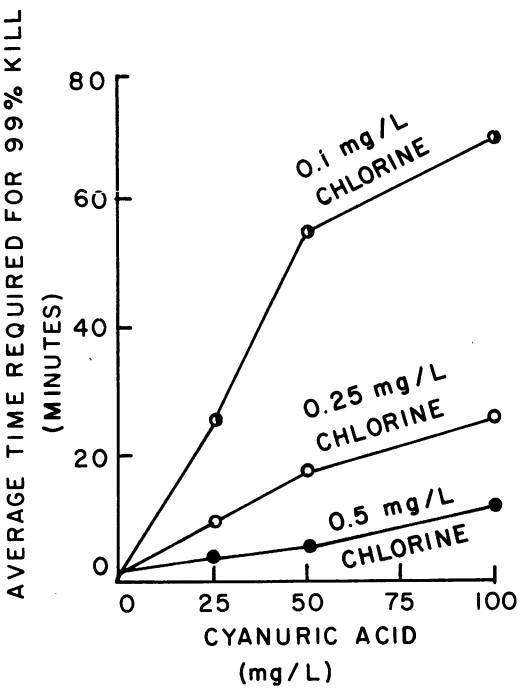

FIG. 4. *Effect of different concentrations of cyanuric acid on the bactericidal properties of 0.1, 0.25, and 0.5 mg of chlorine per liter.*

been well established in the literature. Experiments were carried out to evaluate the effects of ammonia at levels similar to those expected in swimming pools on the bactericidal properties of chlorine and cyanuric acid at the levels that are expected in swimming pools.

Tests on the loss of chlorine from buffered test water showed that there was a gradual decrease in the level of chlorine within the first 5 hr of chlorination, and that this loss amounted to approximately 50% of the added chlorine when 0.5 mg of chlorine per liter was added to inoculated test water (1,000,000 bacteria per ml). However, in the presence of 0.1 mg of ammonia nitrogen per liter from ammonium chloride (NH_4Cl), the loss of chlorine in inoculated test water was less than 20%.

Preliminary tests on the effect of ammonia on the bactericidal properties of chlorine established that the order in which additions are made to test water is of prime importance. Cyanuric acid combined with chlorine immediately, as evidenced by the fact that there was no difference in the rate of kill in media in which the bacteria were added immediately after 25 mg of cyanuric acid per liter was added to chlorinated test water as compared with adding the bacteria 2 min after the addition of cyanuric acid. In contrast, the time required for a 99% kill of bacteria increased from 4 min, when the medium was inoculated immediately after the addition of ammonia nitrogen (0.4 mg per liter) to chlorinated (0.5 mg per liter) test water, to approximately 120 min when the medium was inoculated 2 min after the addition of ammonia. All further tests with ammonia, therefore, were carried out by adding the bacteria after at least a 2-min reaction time between ammonia and the test water.

The effects of the addition of 0.025 to 5 mg of ammonia nitrogen per liter on the bactericidal properties of chlorine (0.5 mg per liter) were studied in both chlorinated buffered test water (*p*H 7.3) and chlorinated Madison city tap water (hardness, approximately 400 mg of $CaCO_3$ per liter; *p*H of tests, 7.6 to 7.9). Both media gave nearly identical results. A summary of the results is presented by the solid line of Fig. 5 as the time required to kill 99.9% or more of the inoculum of *S. faecalis* in the presence of 0.5 mg of chlorine per liter and different concentrations of added ammonia nitrogen.

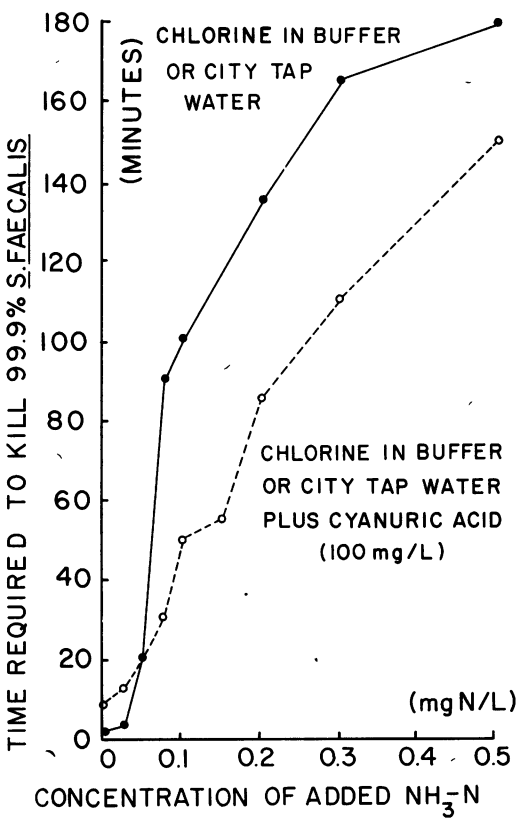

FIG. 5. *Effect of different concentrations of ammonia nitrogen on the rate of kill by chlorine (0.5 mg per liter) or chlorine plus cyanuric acid (100 mg per liter).*

It is evident from the data presented that the addition of as little as 0.05 mg of ammonia nitrogen per liter will cause a decrease in the bactericidal properties of the chlorine, the time for 99.9% kill of the bacteria in these tests increasing from 0.25 min or less to approximately 20 min with the addition of 0.05 mg of ammonia nitrogen per liter. Concentrations of ammonia nitrogen higher than 0.05 mg per liter caused greater losses in the bactericidal properties of solutions of 0.5 mg of chlorine per liter, but the effect was not proportional to the added ammonia at concentrations greater than 0.3 mg per liter.

The effect was determined of the source of nitrogen on the bactericidal properties of 0.5 mg of chlorine per liter. Tests were performed by use of 0.1, 0.5, and 2 mg of nitrogen per liter from the following sources: ammonia nitrogen from ammonium chloride, ammonia nitrogen from diluted secondary sewage effluent, and nitrogen from urea. Ammonia nitrogen from ammonium chloride and diluted sewage effluent had the same effect on the time required to kill *S. faecalis*. However, nitrogen from urea has no measurable effect, as pointed out by Ortenzio and Stuart (12).

Effect of cyanuric acid on the kill rate of chlorine plus ammonia. A series of tests in buffered test water and Madison tap water were carried out to determine the effect of 100 mg of cyanuric acid per liter on the bactericidal properties of chlorinated (0.5 mg per liter) test medium containing different concentrations of added ammonia nitrogen. The results are summarized by the dashed line in Fig. 5 as the time required to kill 99.9% or more of the inoculum of *S. faecalis* in the presence of different concentrations of added ammonia nitrogen.

At ammonia nitrogen levels of less than 0.05 mg per liter, the presence of 100 mg of cyanuric acid per liter required a longer time for a 99.9% kill of the bacteria added than in systems without the cyanuric acid. However, at concentrations of ammonia nitrogen of 0.075 mg per liter and higher, 100 mg of cyanuric acid per liter appeared to decrease the time required for 99.9% kill of bacteria. Related studies have indicated that lesser concentrations of cyanuric acid (25 and 50 mg per liter) have similar but less effective effects on the kill rate of chlorine with *S. faecalis* in the presence of ammonia.

Effect of ammonia in swimming pool waters. Since it had been established that as little as 0.05 mg of ammonia nitrogen per liter could affect the kill rate due to 0.5 mg of chlorine per liter in buffer media and Madison tap water, tests were made with water from five outdoor swimming pools in the Madison area. All of the pools tested had no algal problems at the time of sampling and could be described as having "sparkling clear" waters. The ammonia levels in four of the pools were less than measurable amounts, but one pool had 0.1 mg of nitrogen per liter or more. The latter pool had a high density of young swimmers present for swimming lessons at the three sampling times.

The time required for at least 99.9% kill of *S. faecalis* (1,000,000 per ml) added to membrane-filtered pool waters dechlorinated and chlorinated to 0.5 mg per liter was less than 2 min in the four pool samples with low ammonia levels. However, in three tests with samples from the pool with many children present, the times required for 99.9% kill were 60 min, more than 150 min, and 180 min, depending upon the pool sample. When 0.1 mg of ammonia nitrogen per liter was added to the four samples with low ammonia levels before inoculation with *S. faecalis*, the rate of kill by chlorine was decreased (from 2 to 60 min for 99.9% kill) regardless of the addition of 100 mg of cyanuric acid per liter.

In studies with water from an indoor swimming pool in which a chlorine level of 1 mg per liter or more was apparently maintained, chemical analyses indicated the presence of little or no ammonia nitrogen. Also, three tests in which *S. faecalis* and 0.5 mg of chlorine per liter were used indicated that 99.9% of the added bacteria (1,000,000 per ml) in unfiltered or membrane-filtered pool water were killed within 1 min or less. When ammonia nitrogen (0.3 mg per liter) was added to the dechlorinated pool water and the rate of kill by 0.5 mg of chlorine per liter or chlorine plus cyanuric acid was tested, it was found that in four tests the average time for 99.9% kill of bacteria was 150, 120, and 90 min for chlorine alone, chlorine plus 25 mg of cyanuric acid per liter, and chlorine plus 100 mg of cyanuric acid per liter, respectively. It was apparent, therefore, that ammonia in swimming pool waters had a similar effect to ammonia in buffered, distilled water media.

DISCUSSION

To evaluate the effect of products on the bactericidal properties of chlorine, it is very important that careful control be made of the factors that affect the bactericidal properties of chlorine other than the addition of the products to be tested. Ortenzio (11) pointed out that, if an analyst cannot obtain a specified killing time in the available chlorine control system, the distilled water used in making up the test solution may contain enough ammonia nitrogen to retard the

action of the available chlorine. Thus, the results of tests carried out in a medium that has a chlorine demand cannot be considered to be the same as those obtained with free, available chlorine. Many of the factors affecting the bactericidal action of chlorine have been pointed out by Moore (8), Laubausch (6), and Feng (5).

In the present study, the importance has been demonstrated of washing the bacteria free from chlorine-demand substances carried over from the bacterial growth medium. In an unwashed bacterial suspension, nearly 75% of the chlorine demand of the suspension was shown to be due to such carry-over. This may have been one of the factors involved when some workers (4, 9) reported an incomplete kill of bacteria with 0.5 mg of chlorine per liter within 10 min, whereas other workers (12; Andersen, Ph.D. Thesis) reported kills in 2 min or less. If the rate of kill used as a basis for comparison of bactericides or products affecting bactericides is not the very rapid rate due to free, available chlorine, this fact should be clearly stated, inasmuch as the present data show that competition for chlorine in the system chlorine plus cyanuric acid plus bacteria is very different from that in the systems chlorine plus ammonia plus cyanuric acid plus bacteria and chlorine plus ammonia plus bacteria. In other words, if the chlorine of a system has already combined with a chemical which will affect the bactericidal properties of the chlorine, the effect of adding another product may not cause the same effect on the system as when tested with free, available chlorine.

Since the literature (2, 3, 7) and the present study have indicated that swimming pools can contain ammonia in concentrations shown to affect the bactericidal properties of chlorine, there is certainly some question of the advisability of requiring swimming pool bactericides to provide the same rate of kill as free, available chlorine (10). This would be especially true when the rate of kill due to a practical concentration of a product may be quite significantly more rapid than the rate of kill due to chlorine in the presence of ammonia in swimming pools.

It would appear that, if an objective of sanitarians is a rapid rate of kill of bacteria in swimming pool waters, as much or more concern should be given to the ammonia levels than to the chlorine levels. Studies should be made of the pool operation techniques best suited to remove the ammonia added by swimmers, and recommendations should be given for the use of chemicals not affected by its presence.

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