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Research Article

Isolation, Definition and Chemical Control some of the Bacteria that Cause Contamination of Wall Paintings in Caves

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A B S T R A C T

Background: Caves are unique natural features and habitats where specialized organisms grow. One of the world's main concerns is that of the conservation and preservation of our cultural heritage, including rock art and wall paintings within caves.
Methods: This study was conducted by collecting the samples scraped from wall surfaces at 19 different location in painted caves of Niah cave, Sarawak, and Tempurung cave, Perak. Morphospecies identification and genomic DNA polymorphisms were used to identify the two strains of bacteria. The growth was controlled chemical method using sodium hypochlorite (NaOCl), Calcium hypochlorite $Ca(OCl)_2$ and hydrogen peroxide (H_2O_2).
Results: Morphospecies identification was carried out using a light microscopy and scanning electron microscopy (SEM), both the bacteria, bacteria I and bacteria II were isolated from the soil samples and were Gram-negative bacteria. Based on BLAST search, bacteria I showed 100% with Stenotrophomonas sp (NR 024708.1), and bacteria II showed 100% with *Cryptococcus liquefaciens* (NR 043289. 1). The growth was controlled chemical method using sodium hypochlorite (NaOCl), Calcium hypochlorite $Ca(OCl)_2$ and hydrogen peroxide (H_2O_2). The laboratory studies showed that three chemical were effectively eliminated colonies/cells of the both bacteria compared to the colonies found on the control pate this study.

Keywords: - Niah cave, Tempurung cave, Stenotrophomonas sp, *Cryptococcus liquefaciens*.

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INTRODUCTION

Malaysia is blessed with a large number of natural structures, including caves. These caves are huddled within Malaysia "s rich rainforests and mangrove forests. These caves have become popular archaeological sites as the artefacts found in them can be traced to 40,000 years back, and ancient rock paintings can be found in these caves. There are also other natural attractions such as massive stalagmites and stalactites, which draw the attention of researchers [1]. In Malaysia, Tempurung cave, which is located in Gopeng, Perak; the painted cave, located in the Niah National Park, Sarawak. In caves, different habitats are populated by different microorganisms such as bacteria, fungi, algae and protozoa. It is a known fact that microbes are usually identified in water bodies, rocky surfaces, on sediments and in guano. Another important group of microorganisms are those which develop through different types of interaction with troglobitic animals, e.g. epibionts and parasites in caves [2].

Bacteria are single-celled, prokaryote microorganisms normally a few micrometres in length and have a wide range of forms, from spheres to rods and spirals [3]. They can be responsible for the destruction of cultural heritages. Bacterial isolates from indoor environments like caves, churches, museums and chapels, are often attributed to the genus Bacillus or are part of the bacterial population of known or unknown species [4,5], or are Proteobacteria or Actinobacteria. In a larger study to assess microbial communities in the

UNESCO World Heritage Naracoorte Caves [6], it was found that bacteria play critical roles in the formation and biogeochemical cycles of caves, with adverse effects on the environment in the caves. Also visiting bat caves. May lead to infection of cases may occur because of the disturbance of soil contaminated with *H. capsulatum* [7]. Bacterial isolates have commonly been ascribed to the genus Bacillus. A study by [8] used DNA-based techniques to characterize the bacterial community and the study revealed that a significant part of the bacterial population is made up of unknown species.

Malaysia enjoys wonderful caves of beauty and breadth, but these natural resources are exposed to the growth of undesirable microorganisms, which grow on the walls and ceilings of all caves, due to naturally occurring levels of nutrients and moisture that are sufficient to support the growth of undesirable microorganisms. This growth degrades the natural beauty of cave walls, and threatens their archaeological value. Therefore, an attempt should be made to eliminate these undesirable microorganisms. Objective of the study to isolation and identify of the bacteria inhabiting the cave walls of Tempurung and Niah caves that cause damage to the rock art and wall painting.

MATERIALS AND METHODS

Study Area: The area selected for this study was the Niah Caves, situated in Miri, Sarawak to the north of Borneo, Malaysia (3.802 N-113.773 E). Tempurung Cave the Tempurung Cave is situated in Gopeng, Perak.

Isolation, purification and identification of bacteria

Isolation of bacteria, the samples were passed through a sieve (1.7 mm mesh) to get rid of large pieces of debris and vegetation. Five grams of the samples from wall paintings was added to 100 ml of nutrient broth (NB) in sterilized 250 ml Erlenmeyer flasks. The cultures were incubated overnight in a shaking water bath at 37 °C (24 h) at 180 rpm. The bacteria were originally isolated by plating dilutions of the sample in (NB). One ml portions from each dilution, ranging from 10^{-1} to 10^{-5}, were spread onto the Nutrient Agar (NA) and incubated at 37 °C for 48 hours. The developed colonies were picked and purified by streaking them on the NA plates. The bacterial isolates were kept on the NA plates at 4 °C and re-cultured every 3 weeks [9].

Observation of the isolated bacteria

Observation using light microscopy

In order to examine microorganisms under a light microscopy at magnification of 10x, 20x, 40x or/and 100x, a drop of solution containing the bacteria, or a single colony (picked up using a sterile toothpick or loop) was placed on a clean microscope slide. The cover slip was lowered and tilted at an angle, until the lower edge touched the slide at the edge of the microorganism solution drop [10].

Gram staining was conducted; The Gram stain procedure enables bacteria to retain the colour of the stains, based on the differences in the chemical and physical properties of the cell wall. Gram-positive results in a purple-blue colour, while Gram-negative results in a pink-red colour. Observations of live microorganism cells on the worksheet include the nucleus, colour, size and cell shapes[11-14].

Observation using scanning electron microscopy (SEM)

The hexamethyldisilazane **(**HMDS method) method was used to prepare the cells of bacteria grown in the liquid media. The samples were centrifuged 4,000 rpm to obtain pellet. After centrifugation, the supernatant was discarded and the pellets were re-suspended using the McDowell-Trump fixative, which was prepared in 0.1 M phosphate buffer (pH 7.2) for at least 2 hours. The samples were centrifuged again and the pellets obtained were transferred into Eppendorf tubes. The pellets were then re-suspended in 0.1 M phosphate buffer (buffer wash 1). Similar steps were repeated for buffer wash 2. After buffer wash 2, the pellets were re-suspended into osmium tetroxide prepared in the phosphate buffer for 1 hour (post-fixation). The post-fixation process was repeated twice, followed by a dehydration process with 50% ethanol for 10 minutes, 75% ethanol for 10 minutes, 95% ethanol for 10 minutes, 100% ethanol for 10 minutes (x2), and Hexamethyldisilazane (HMDS) for 10 minutes. After dehydration, the HMDS was transferred from the tube, and the tube with the cells was left in a desiccator under room temperature. Then the dried cells were mounted onto an SEM specimen stub with double-sided sticky tape (or silver paint). The specimen was covered with gold, gold/palladium, chromium or carbon, and observed under the SEM [15].

Genomic DNA extraction

For DNA extraction, phenol chloroform extraction method was used. In this method, 50 ml of the culture pellet from the exponential phase was transferred into an Eppendorf tube and centrifuged at 14000 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended with 500 µL of Sucrose Tris EDTA (STE) solution, pH 7.4. Then, 50 µL of 10% sodium dedocyl sulphate (SDS, also called sodium dodecyl sulphate) was added and incubated at 37 °C in a water bath for one hour. After that, 3µL of proteinase K was added and incubated again under the same conditions[16,17]. Next, phenol: chloroform extraction was performed by adding equal volumes of phenol and chloroform before centrifuged at 14000 rpm for 10 minutes. The bottom layer was discarded and the process was repeated three times. Then, chloroform extraction was performed by adding an equal volume of chloroform and centrifuged at 14000 rpm for 10 minutes. The bottom layer was discarded and the process was repeated twice. Then, the upper layer was precipitated with absolute ethanol and was incubated overnight at -20 °C. The Eppendorf tube was centrifuged at 14000 rpm for 10 minutes. The pellet was re-suspended with 70% ethanol and centrifuged at 14000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. The tube was given a short spin and the supernatant was discarded. The DNA pellet was re-dissolved in 30 µL of TE buffer/ dH_2O. It was stored in small aliquots at 4 °C until further use.

PCR Amplification of ribosomal DNA and sequencing

The amplification of 16S was performed for bacteria was carried using a set of 16S primers (16SF 5"

TNANACATGCAAGTCGAGCG 3", 16SR 5" ACGGGCGGTGTGTGTAC 3"). PCR was conducted using 50µL reaction containing 0.5µL of DNA template, 1.5µL of 50mM Magnesium chloride, 5.0µL of 10X PCR buffer, 1.0µL of 10 mM dNTP mix, 2µL of 25 pmole of 16S primers, 2µL of 5µ/µL *Taq* DNA polymerase and 37.37µL of deionized distilled water to make up a total volume of 50µL. The PCR amplification was performed as follows: 5 minutes for initial denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 1 minute 40 seconds, and a final extension at 72 °C for 5 minutes. The PCR products were then checked on 1% agarose gel. The desired bands were excised from the agarose gel and purified by using the Intron Fragment and DNA Gel Extraction Kit (Intron, Korea) according to the manufacturer"s instructions. The purified products were sent to a service provider for DNA sequencing.

Pair- Wise Sequence Alignment

The sequences of bacteria were aligned using Clustal W included in Molecular Evolution Genetic Analysis (MEGA S), and the consensus sequences were used to compare with other sequences in the Gen Bank (http://www.ncbi.nlm.nih.gov /genbank/) using BLAST.

Preparation of chemical agents to control the growth of bacteria

Three chemical agents used as disinfectant were tested, which were sodium hypochlorite (NaOCl), Calcium hypochlorite Ca $(OCl)_2$ and hydrogen peroxide (H_2O_2). The solution was diluted to give accurate concentrations of 0, 5, 10, 15, 30 and 60 % to be tested against the bacteria. Table 1 shows how concentrations of chemicals (%) were prepared.

Table 1: Preparation of chemical concentrations (%) (Sodium hypochlorite (NaOCl), Calcium hypochlorite Ca $(OCl)_2$ and hydrogen peroxide (H_2O_2)

0%	5%	15%	30%	60%
100 ml YM broth with culture from exponential phase	5 ml chemical agents + 95 ml nutrient broth (NB) with culture from exponential phase	15 ml chemical agents + 85 ml nutrient broth (NB) with culture from exponential phase	30 ml chemical agents + 70 ml nutrient broth (NB) with culture from exponential phase	60 ml chemical agents + 40 ml nutrient broth (NB) with culture from exponential phase

Preparation of bacteria inoculum and exposure to test chemical

1. **Test media:** NB and NA were used for cultivation of bacteria.
2. **Inoculum preparation:** Four or five colonies were transferred to 100 ml of specific media to obtain a suspension equivalent to the turbidity of a McFarland 0.5 standard, which can be used to determine approximate numbers of bacteria in the experimental tubes.
3. **Incubation**: Incubation took place on a mechanical shaker at 35°C until the suspension was visibly turbid (the growing microorganisms were not allowed to grow to produce heavy turbidity). This preparation should correspond to an early to mid-logarithmic growth phase for more rapidly growing bacteria. The growth phase was confirmed by measuring the growth by observing the spectrophotometer at 600 nm wavelength for bacteria.
4. **Preparation of chemical reagent working concentrations:** Referring to Table.1, 5 ml of a chemical reagents [(NaOCl), $Ca(OCl)_2$ or H_2O_2] was added to 95 ml of the media with culture from the exponential phase ($6x10^5$cfu/ml) to obtain a concentration of 5%; 15 ml of a chemical agent [(NaOCl, Ca $(OCl)_2$, or H_2O_2)] was added to 85 ml of specific media with culture from the exponential phase ($6x10^5$cfu/mL) to get a concentration of 15%; 30 ml of a chemical agent was added to 70% of the media with culture from the exponential phase ($6x10^5$cfu/mL) to obtain a concentration of 30%, and 40 ml of a chemical agent was added to 60% of specific media with culture from the exponential phase ($6x10^5$cfu/mL) to obtain a concentration of 60%. The control sample comprised 100% of specific media without chemicals, inoculated with culture from the exponential phase ($6x10^5$cfu/ml). The final bacteria concentration for the experiment was approximately $6x10^5$cfu/ml.
5. Chemical agent working concentrations (0, 5, 15, 30 or 60 %), with approximately $6x10^5$cfu/ml of bacteria in the test tubes, were left for an incubation period up to 120 minutes.
6. After 1, 2, 5, 10, 15, 30, 60, 75 and 120 minutes of incubation, 30µL of the chemical agent working concentrations containing the bacteria in the test tubes were transferred into an agar plate (with specific media).
7. 30µl of the chemical agent working concentrations was transferred to agar after every exposure. Then all the Petri dishes were incubated for 24 to 48 hours at 35 °C for the cultures to grow and the cfu per ml versus time graph was plotted. Then the colonies on each of the serial dilution plates showing 30 to 300cfu were counted. The data were recorded on a worksheet.
8. **Statistical analysis:** A scatter chart was inserted to compare pairs of values. The data were analyzed using a general linear model (univariate) (SPSS, 20) to test for possible significant differences in the independent variables (concentration, time and chemical agents or physical control). A simple main effects analysis followed by Tukey"s multiple comparison test (Tukey's HSD, honest significant difference test) were utilized, when appropriate, as post-hoc procedures to follow up on the significant main effect and interactions. A p-value of less than 0.05 ($P< 0.05$) was considered significant[18-21].

RESULTS

Isolation and Identification of bacteria

Two types of bacteria, Bacteria I and Bacteria II were isolated from the Malaysian caves. Both the bacteria were isolated from the soil samples and were Gram-negative bacteria. Morphospecies bacteria I is a genus of Gram-negative bacteria (Plates 1 and 2), Morphospecies bacteria II is a genus of Gram-negative bacteria (Plate 3 and 4).

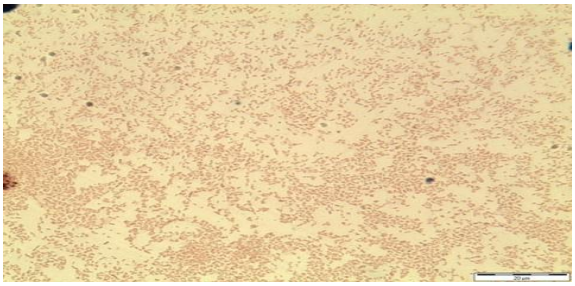

Plate 1: Bacteria I: under light microscope a genus of Gram-negative bacteria.

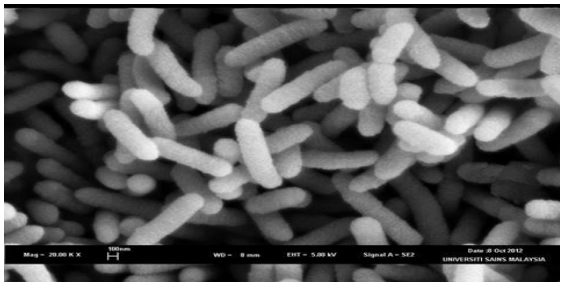

Plate 2: Bacteria I: under SEM observation.

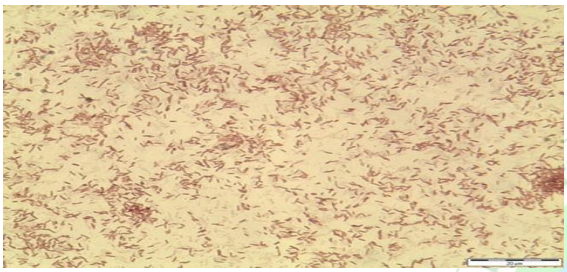

Plate 3: Bacteria II: under light Microscope, a Gram-negative.

Plate 4: Bacteria II: under SEM observation

Molecular characterization

The cells of bacteria were harvested through centrifugation and their genomic DNA was extracted (in duplicate). Samples with DNA bands that showed high molecular weights and brightness were selected for PCR analysis. The molecular weights obtained for PCR products were 1364bp for Bacteria I, 1372bp for Bacteria II. According to BLAST search, bacteria I showed 100% with Stenotrophomonas sp (NR 024708. 1), bacteria II showed 100% with *Cryptococcus liquefaciens* (NR 043289. 1).

Chemical Control

Three disinfectant, NaOCl, Ca $(OCl)_2$ and H_2O_2 were evaluated for their ability and effectiveness in controlling growth of the bacteria in the present study.

Sodium hypochlorite (NaOCl)

Results showed that NaOCl reduced the number of bacteria to zero in all concentrations (Figure 1) at all exposure times. Exposure to 5% NaOCl concentration for one minute was enough to eliminate *Stenotrophomonas* sp. and *Pseudomonas aeruginosa*.

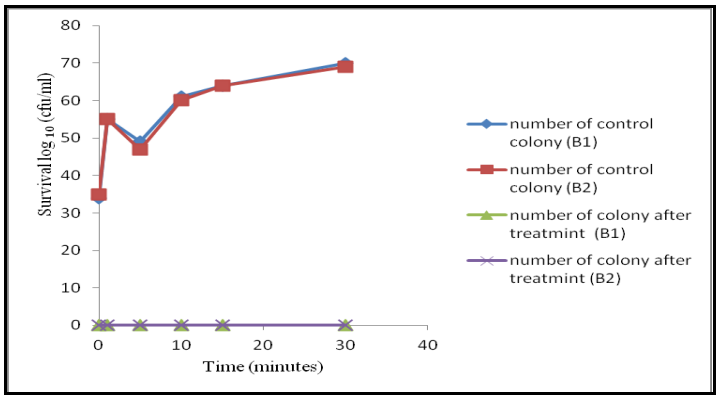

Figure 1: There was no growth of bacteria after exposure to sodium hypochlorite (NaOCl) *Stenotrophomonas*sp (B1). And *Pseudomonas aeruginosa* (B2).

Calcium hypochlorite Ca $(OCl)_2$

Growth of *Stenotrophomonas* sp. and *Pseudomonas aeruginosa* were reduced to zero for all concentrations tested at all exposure duration (Figure 2).

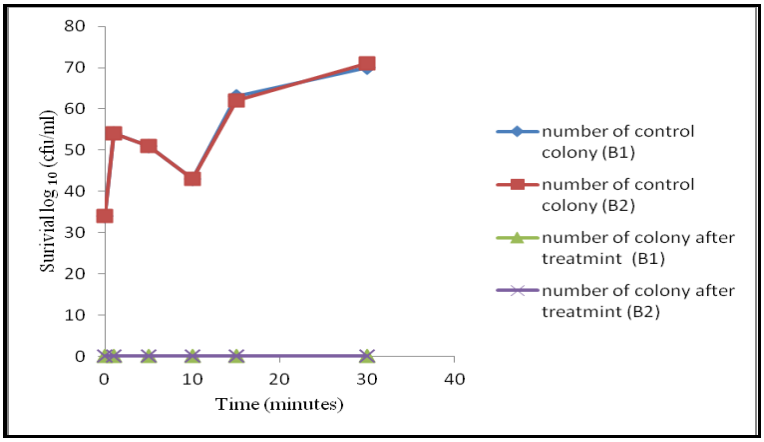

Figure 2: there was no growth of bacteria *Stenotrophomonas* sp. (B1) and *Pseudomonas aeruginosa* (B2) after exposure to calcium hypochlorite Ca $(OCl)_2$.

Hydrogen peroxide (H_2O_2)

The results showed that H_2O_2 effectively reduced the number of bacteria to zero at all concentrations (Figure 3). Treatment with 5% concentration for one minute was enough to eliminate colonies of *Stenotrophomonas* sp. and *P. aeruginosa*.

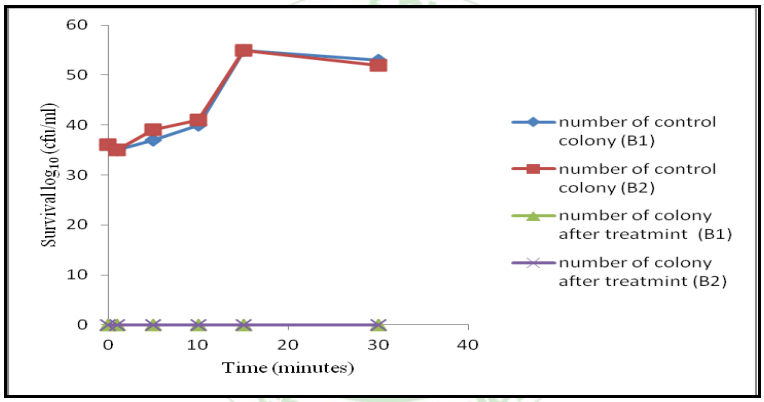

Figure 3: There was no growth of bacteria *Stenotrophomonas* sp. (B1) and *Pseudomonas aeruginosa* (B2) after exposure to hydrogen peroxide (H_2O_2).

DISCUSSION

A cave is a place characterized by low temperature and humidity, which is more or less constant throughout the year [22]. That made Tempurung and Niah Caves a suitable environment for the invasion of bacteria that appear as a result of the presence of bats, insects and some reptiles that live in the cave. The occurrence of Gram-negative bacteria in the cave such as *Stenotrophomonas* sp. and *Pseudomonas* sp. was also reported by[23], where Gram-negative bacteria made up of 81% of lampenflora in Magura Cave, Bulgaria. Subterranean caves are characterized by almost stable temperature with high humidity and these factors favor the growth of heterotrophic bacteria, from which actinomycetes predominate[24]. In some cases, *Streptomyces* species are particularly found to be abundant.

The two species of bacteria, *P. aeruginosa* and *Stenotrophomonas* sp. and the three species of yeasts *P. guilliermondii*, *C. liquefaciens* and *R. dairenesis*, based on the dry weight and cell enumeration of *Synechococcus* sp. and *Micractinium* sp. showed that these microorganisms were able to grow together. This shows that no matter how varied the conditions inside the caves, these microorganisms thrive in their natural growth[23, 25].

The bacteria recorded in the Tempurung and Niah Caves, partly corresponded to the species found in other caves such as Leontari Cave, Ponikve cave, Sybil"s cave, bat-inhabited cave in Japan, and Magure cave[25-28].

Cave is one of the environments with the factors that make the cave a unique environment: 1) the absence of sunlight, 2) the stability of the microclimate of the cave because of the surrounding rock layer which isolated the cave from the external weather conditions, 3) rich in nutrients contributed by guano, birds and animal droppings that support the growth of lampenflora. Some microflora grows on wood, animal bones and on the cave walls, ceiling and floor. These organisms thrive in this environment, particularly in the caves with artificial illumination. High levels of phosphorus and nitrogen in guano, high humidity in the range of 85% to 98% and sufficient light whether natural or artificial, enhance the colonization and growth of microorganisms such as bacteria, cyanobacteria and protozoa[2,29].

The results showed that treatment with 5% concentration of sodium hypochlorite (NaOCl) effectively eliminated colonies/cells of bacteria compared to the colonies found on the control pate this study, 30 minute treatment with 15% NaOCl concentration was enough to eliminate the entire lampenflora isolated. Treatment with 5% NaOCl concentration was enough to destroy *Stenotrophomonas* sp. and *Cryptococcus liquefaciens*. According to a study conducted by[30], NaOCl was proven to be an efficient biocide. Similar results were also reported by[20], where NaOCl at concentrations of 5% and 10% were extremely effective in eliminating the lampenflora tested.[30] and[31] also found that 5% of NaOCl was sufficient to destroy the entire lampenflora. Based on the present and previous results reported, NaOCl (even at the lower concentration) is recommended as an effective disinfectant. In the present study, 5% concentration of H_2O_2 reduced the number of cells of *Stenotrophomonas* sp. The result by[32], who found that at 5% concentration of H_2O_2 was enough to eliminate their entire lampenflora in the laboratory. Calcium hypochlorite $Ca(OCl)_2$ was effective in eliminating all the microorganisms tested. The results showed that treatment with 5% concentration of Ca $(OCl)_2$ was enough to reduce and eliminate *Stenotrophomonas* sp. and *C. liquefaciens*. Fifteen percent concentration of $Ca(OCl)_2$ effectively eliminated *Stenotrophomonas* sp. at the first few minutes of exposure. These results support the previous report by[20] whereby calcium hypochlorite was effective in eliminating the lampenflora at low concentrations (5%, 10%).

However, the treatment with Ca(OCl)2 and NaOCl may be accompanied by some problems such as the smell of chlorine or problem of percolation and explosive reaction with ammonia, amines or organic sulphides; although it is considered a good chemical to eliminate the bacteria.

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