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Isolation and Selection of Bacteria Chemotactic to Chlorobenzene and Other Organic Chlorinated Compounds

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Abstract: Nowadays, polluting compounds are commonly present in the environment, which seriously affect human's health. However, the current methods for detecting these compounds are costly, expertise-requiring and technically complicated as well. Thus, in this work, we studied the applicability of the chemotactic responses of bacteria toward some popular polluting organic chlorinated compounds (e.g. chlorobenzene) in order to develop a biological method that is simple, economical, and time-saving to detect those compounds in environmental samples. From 169 bacterial strains isolated from different national parks such as Cuc Phuong, XuanThuy and Tam Dao, three bacterial strains (HTD 3.8, HTD 3.12 and HTD 3.15) having the capability of negative chemotaxis towards chlorobenzene could be selected. Among them, HTD 3.8 displayed a better response to chlorobenzene, with a threshold concentration of approximately 0.3M. After testing the chemotactic responses of HTD 3.8 to several aromatic and/or chlorinated compounds, we discovered a high specificity of the responses of HTD 3.8 to molecules harbouring the functional group of –C-Cl (including also trichlomethane). Furthermore, conditions for the assay were optimized by investigating the chemotactic responses of HTD 3.8 in different minimal soft-agar media with different temperatures, NaCl concentrations and pHs. According to 16S rRNA gene sequencing result, HTD 3.8 is the most closely related to a *Pseudomonas* sp. The result of an initial experiment using trichloromethane as a competitive ligand suggested some possible chemotactic receptors of HTD 3.8 that are responsible for sensing –C-Cl containing compounds.

Keywords: Negative chemotaxis, chlorobenzene, organic chlorinated compounds.

1. Introduction

Socio-economic developments lead to adverse negative impacts to human beings. Through the industrialization and human daily activities, the amount of organic compounds used has been dramatically soared. Since the industrial wastes are persistently decomposed into environmental pollutants, it is not possible to ignore the organic halogen compounds such as trichloroethylen, trichloromethane, dichlorodiphenyltrichloroethane (DDT), chlorobenzen, and many others. They are usually produced as waste in the oil refining process and the manufacture of medical equipment, medicines and plant protection products. As a consequence, they accumulate with time in soil and sediments, causing water pollution, and thus physiological disruptions

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and cancer diseases if in contact with humans. One of the earliest organic chemical compounds that have been produced in large quantities is chlorobenzene (CLB) or monochlorobenzene. A CLB molecule consists of a benzene ring that links to a chlorinated group. The greatest application of CLB is in the organic chemical manufacturing industry, and the manufacture of dyes, insecticides or solvents [1, 2]. After being released, CLB enters the human body through various ways such as inhalation, drinking or direct contact with skin. As a consequence, this leads to drowsiness, incoordination and unconsciousness or negative effect on liver, kidney and lung damages [2].

The detection of chlorobenzene as well as other organic halogen compounds in the environment in order to reduce their harmful effects is therefore very essential and has been deployed strongly in global scale. Some popular methods that have been used so far for the detection are chromatography, spectroscopy, mass spectrometry [3], and the uses of optical sensors [4] or biosensors, purge-and-trap collection, etc. The most efficient and accurate method of detection is chromatography (high performance, liquid chromatography, gas chromatography [5], thin layer chromatography etc.). Even though the advantages of using this method include a fast detectability, higher accuracy and better detection limits, this method also requires sophisticated techniques, advanced equipment and high cost. Beside the detection by using chemical and physical methods, scientists are focusing on approaches using biological measures – which are more environmentally friendly and effective. In particular, the use of microorganisms that are capable of detecting organohalogens by chemotaxis can be regarded as a promising method in the future and thus deserves to be thoroughly studied [6,7].

Bacterial populations may encounter a large spectrum of environmental conditions during their life cycles. Due to their small sizes and relative simplicity, their ability to adjust the environment to their needs is very limited. Instead, they apparently adopted a strategy of moving from one environment to another environment. Chemotaxis also serves as a cell-to-cell communication and cell recruitment under appropriate stress conditions. In general, there are two types of chemotaxis, including negative chemotaxis when target chemicals serve as a chemorepellent stimulus and positive one when chemicals are chemoattractants [8, 9].

This research aims to seek for microorganisms which are chemotactic toward chlorobenzene and some other chlorinated compounds in the environment and subsequently exploring their chemotactic mechanism. Our ultimate goal is to develop a method for the detection of the pollutants that are structurally similar.

2. Materials and Methods

Organism and culture media

The organisms used for this study were isolated from natural soil sources in Tam Đảo National Park (HTD strains), and natural muddy sources in Cúc Phương National Park (CP strains) and Xuân Thủy National Park (XT strains) by culturing on Luria Broth medium (containing 16g agar, 5 g NaCl, 10 g Peptone and 5 g extract yeast / litre) for growing under surrounding temperature of 30 ^{o}C.

Semi-solid agar gradient method for chemotaxis tests

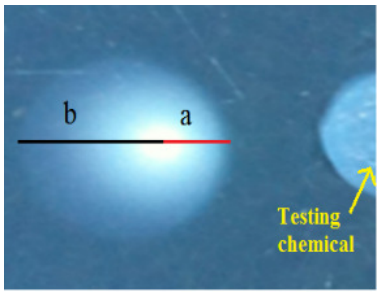
In order to select bacteria that have the capability of negative chemotaxis toward tested chemicals, including chlorobenzene, an assay based on the use minimal semisolid agar medium was applied. A liter of minimal semisolid agar medium contained 0.2 g agar, 0.5 g NaCl, 1.47 g $K_2PO_4.3H_2O$, 0.48 g KH_2PO_4 and 0.132 g $(NH_4)_2SO_4$, followed by sterilization and with additional of the following components through bacteria membrane filter: 0.246g $MgSO_4.7H_2O$, 0.01ml Thiamine HCl and 0.0815 ml Glycerol. After

preparing the medium, a 10 diameter 2% agar plug containing the tested chemical (at the concentration to be tested) was put on the center of each medium plate. Sterile toothpicks were used to stab fresh test bacterial cells from pre-grown cultures into test plates at a 2-centimeter distance from the plate centre. After about 16-20 hours of incubation at 30°C, the chemotactic responses of the test bacteria to the test chemicals were assessed [10].

Growth inhibition test

To clarify whether the results of the semisolid agar test were really due to negative chemotaxis or only due to inhibition of growth, the authors used hard agar (2%) containing the same minimal medium for culturing the test bacteria by spreading on plates. A 100 µL suspension containing an overnight culture of each bacterial strain of interest in LB broth was evenly spread onto the agar surface of a Petri plate. Subsequently, an agar plug containing the test chemical, e.g. chlorobenzene, at the concentration to be tested, was placed onto the center of the plate, and the plate was incubated for 16-20 hours at 30 °C.

Chemotactic response sensitivity test



$$i = \frac{a}{b} - 1$$

In which:

i: Chemotactic index

a: The distance from the closest edge to the centre of the colony

b: The distance from the furthest edge to the centre of the colony

Chemical concentration is also one of the factors adversely affecting bacterial chemotaxis [15]. In order to find the threshold concentration at which a bacterial strain of interest starts to show its response of negative chemotaxis, semisolid agar tests were carried out with different concentrations of chlorobenzene, ranging from 0.02 M up to 1 M. We used "chemotactic index" which is illustrated by the following formula in order to estimate on the capability of chemotaxis.

Chemotactic response specificity test

Semisolid agar test and growth inhibition tests were repeated to test the chemotactic responses of the selected strain to several benzene-ring-containing compounds (e.g., phenol, aniline, toluene, sodium benzoate) and chlorinated ones (e.g., trichloroethylene (TCE) and trichloromethane (TCM)).

Competitive chemotactic ligand test

Semisolid agar method with minimal medium containing 0.005M trichloromethane (TCM) (instead of chlorobenzene) was used to test the effect of this possible competitive ligand on the negative chemotactic response of the selected bacterium toward chlorobenzene.

3. Results

Selection of bacterial strains having chemotactic responses to chlorobenzene

From 169 isolated bacterial strains and by using the minimal semisolid-agar method, we discovered 5 bacterial strains (HTD 3.8, HTD 3.12, HTD 3.15, CP 1.8 and CP 10.3) whose colonies developed away from the chlorobenzene-containing agar plugs (Fig.1). However, the results of growth inhibition tests strongly indicated that the response of CP 1.8 was due to growth inhibition by chlorobenzene (data not shown), while other strains (HTD 3.8, HTD 3.12, HTD 3.15 and CP 10.3) were

actually chemotactically repelled by chlorobenzene.

Chemotactic response sensitivity

HTD 3.8, HTD 3.12 and HTD 3.15 were tested for their response sensitivity with chlorobenzene concentrations ranging from 0.02M up to 1M. The strains showed very weak positive responses or no response to chlorobenzene at lower concentrations (less than 0.4 M) of chlorobenzene, whereas at higher concentrations, they show clear negative chemotactic responses (Fig. 2). The response curve of HTD 3.8 shows that the strain has the most consistent capability and a response threshold of approximately 0.3M chlorobenzene. Therefore, we decided to use HTD 3.8 for the further experiments

Chemotactic response specificity of HTD 3.8

By considering that the molecular structure of chlorobenzene has a benzene ring linked to a chlorinated group, we further carried out experiments in order to find out potential chemical groups responsible for the negative chemotactic ability toward chlorobenzene of the selected bacterial strain HTD 3.8.

Responses to other aromatic compounds: According to the results of both semisolid agar test and growth inhibition test, HTD 3.8 appeared repelled by phenol but this turned out to be due to the growth inhibition (Fig. 3). In contrast, other aromatic compounds (aniline, toluene, sodium benzoate) did not show their chemotactic responses in semisolid-agar medium.

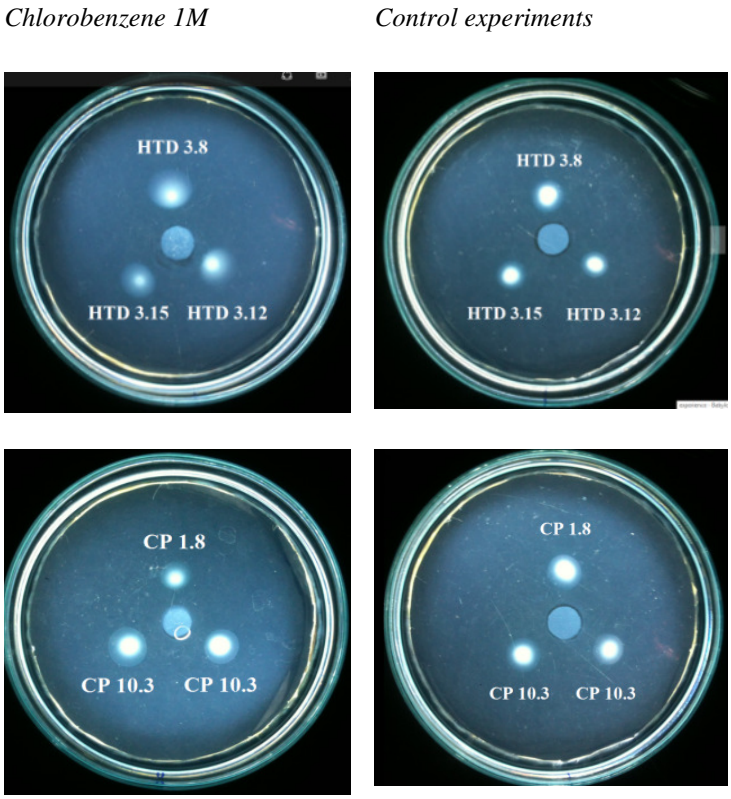


Figure 1. Five bacterial strains whose colonies tend to develop away from chlorobenzene while colonies in control experiments are round.

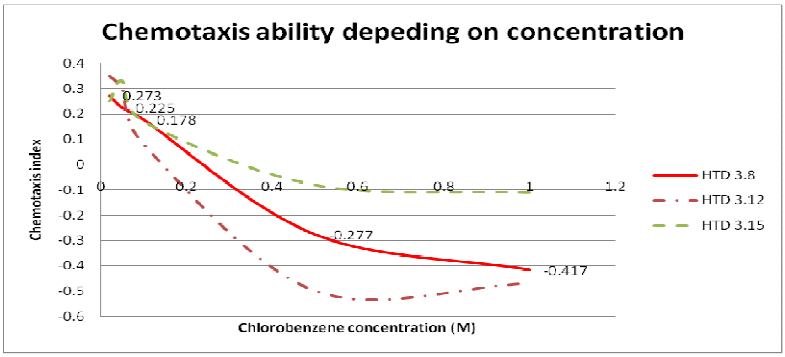


Figure 2. Chemotactic responses of the bacterial strains in relation to the chlorobenzene concentration.

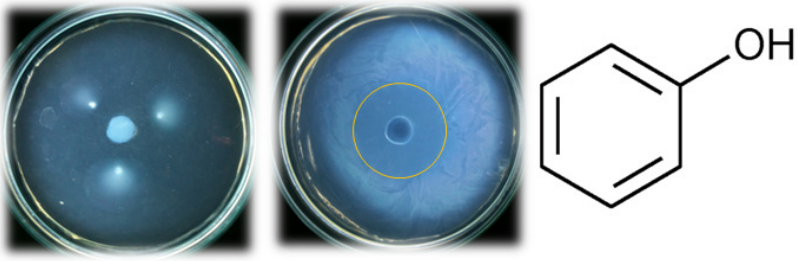


Figure 3. The results of testing the chemotactic response of HTD 3.8 to phenol and the effect of phenol on its growth.

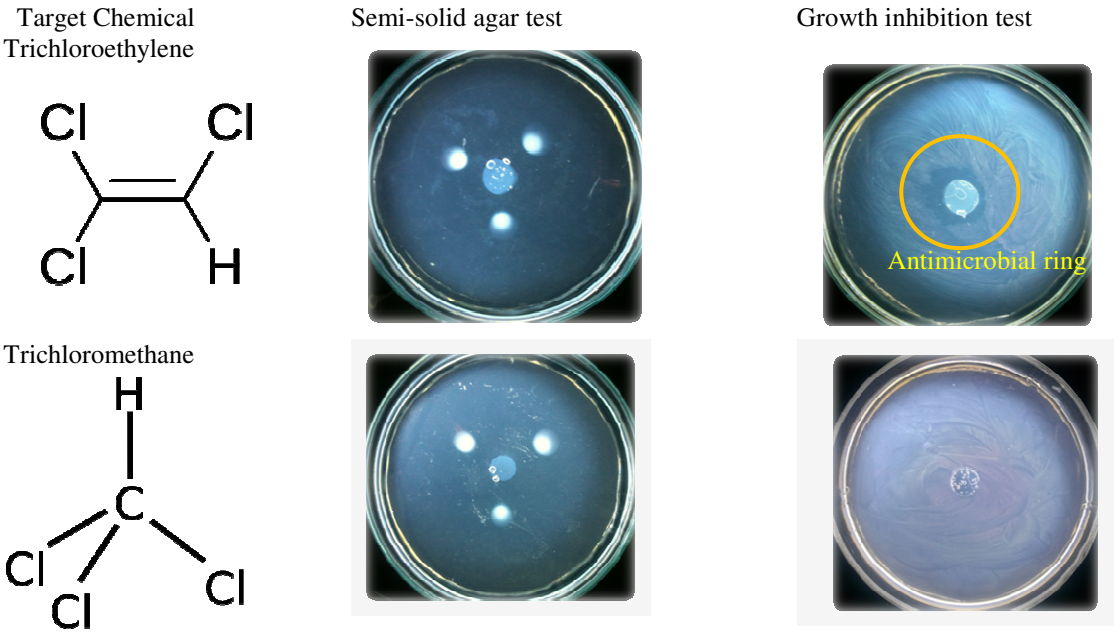


Figure 4. Chemotactic behaviours (left) and growth (right) of HTD 3.8 in response to the presence of two compounds containing the –C-Cl group. Notes: The chemical formula of the two compounds are highly similar.

Effect of environmental conditions on the negative chemotatic activity of HTD 3.8 toward chlorobenzene [11-13]

In this study, it is reasonable that at 1% NaCl concentration, the colonies of HTD 3.8 strain swarmed and showed the strongest chemotactic ability, while those at 3% NaCl were the smallest and swarmed the most slowly (Table 1).

Table 1. Effect of environmental conditions on negatively chemotactic activities

NaCl Concentration	0.5%	1%	2%	3%
	+++	++++	++	+
pH	4	7	9	
	-	++++	++	
Temperature (^oC)	10	20	30	
	-	++	++++	

Notes: -: no response; +: weak response; ++: relatively weak response; +++: strong response; ++++: very strong response

Same experimental works were properly set up to test the effect of temperature. At low temperatures (10 and 20^{o}C), the colonies were small and unable to swarm, in contrast to those at higher temperature (30^{o}C). This significant change indicates that low temperature has a considerable effect on the movement as well as the chemotactic capability of HTD 3.8.

Among three different pHs (4, 7 and 9), HTD 3.8 was almost unable to grow in the acidic environment (pH 4) but develop dramatically in neutral environment (pH 7).

Identification of HTD 3.8

Morphological observations strongly confirmed that the HTD 3.8 strain is a Gram-negative bacterium with rod-shaped cells (Fig. 5)

The 16S rRNA gene fragment of HTD 3.8 was successfully amplified (data not shown). Sequencing analysis of this gene fragment showed a 96 % similarity with the 16S rRNA gene fragment of *Pseudomonas aeruginosa*.

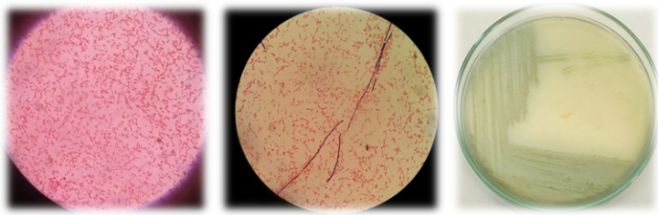


Figure 5. Colonies and cells of the HTD 3.8 strain isolated from LB medium.

All the results above suggested that the strain is probably a novel *Pseudomonas* species but this requires further investigation.

The chemotactic receptor that may be responsible for chlorobenzene chemotaxis of HTD 3.8

A lot of others previous researches related to the chemotaxis of *Pseudomonas aeruginosa* [14, 15] have illustrated clearly that *P. aeruginosa* was repelled by TCM as well as TCE and this negative chemotatic response toward these chemicals was executed by three methyl-accepting chemotactic proteins (MCP): PctA, PctB and PctA [16, 17]. Thus our hypothesis is that HTD 3.8 in this study might also execute its chemotactic activity to chlorobenzene by using the same chemoreceptor(s). In order to initially prove this, we tested whether TCM could function as a possible competitive ligand to chlorobenzene by assessing the chemotactic response of HTD

3.8 on a semi-solid minimal medium agar containing 0.005 M of TCM.

In the medium containing TCM, the chemotactic capability of HTD 3.8 toward chlorobenzene was weaker than that in the medium without TCM (Fig. 6). It is therefore suggest that TCM could be a competitive ligand to chlorobenzene in the negative chemotaxis of HTD 3.8.

4. Discussion

This research has demonstrated that HTD 3.8 is capable of chemotactically responding to chlorobenzene as well as to trichloromethane. The tested chlorobenzene concentration was 0.5 M which is higher than the maximum level of chlorobenzene in drinking water (0.1ppm) [18]. As a result, negative chemotaxis of bacteria and growth inhibition could be clearly observed at this concentration.

According to our results, it is undeniable that environmental conditions such as salt concentration, pH, temperature, etc. have considerable effects on the chemotactic capability of HTD 3.8 strain. With the same amount of chlorobenzene, the differences in experimental conditions will results in different swimming consequences, leading to different chemotactic responses. These results are also similar to those of other previous studies on the influence of environmental conditions on the bacterial mobility as well as the capability of bacterial chemotaxis [3, 4]

Furthermore, the reduced response of HTD 3.8 to chlorobenzene when this organism was tested in semisolid trichloromethane-containing medium is consistent and could be explained by the competition of ligands to interact with trichloromethane chemoreceptors [17].

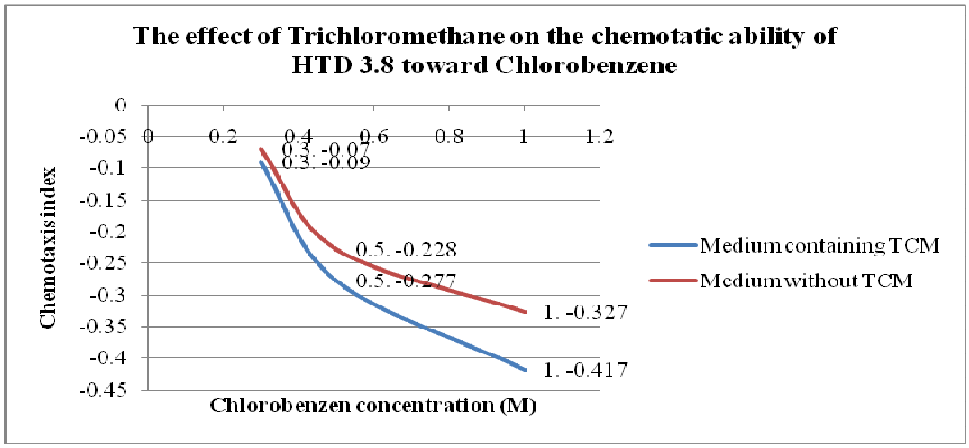


Figure 6. Chemotactic ability of HTD 3.8 toward Chlorbenzene in the medium containing TCM.

5. Conclusion

In this study, we have successfully isolated a bacterial strain, HTD 3.8 from the soil sample in Tam Đảo National Park, which is repelled by chlorobenzene with a threshold concentration of approximately 0.3 M. In the medium with 1% NaCl, 30°C and pH 7, the chemotactic capability of HTD 3.8 is the highest. The results of this research can be a prerequisite for the further development of microbial assays for detecting chlorinated organic pollutants.

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Phân lập và tuyển chọn vi khuẩn có khả năng hóa hướng động đến chlorobenzene và một số hợp chất hữu cơ chứa clo

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Tóm tắt: Ngày nay, các hợp chất gây ô nhiễm đang tồn tại phổ biến trong môi trường, gây hại cho sức khỏe con người. Tuy nhiên, các phương pháp hiện nay để phát hiện các hợp chất này đòi hỏi chi phí cao, kỹ thuật phức tạp và cần có các chuyên gia thực hiện. Vì vậy, trong nghiên cứu này, chúng tôi

hướng tới phát triển một phương pháp sinh học đơn giản, kinh tế, tiết kiệm thời gian, có khả năng phát hiện các hợp chất ô nhiễm trong mẫu môi trường bằng việc áp dụng phản ứng hóa hướng động của vi sinh vật tới một số hợp chất hữu cơ chứa clo như chlorobenzene. Từ 169 chủng vi khuẩn được phân lập từ các vườn quốc gia khác nhau như Cúc Phương, Xuân Thủy và Tam Đảo, chúng tôi phân lập và tuyển chọn được ba chủng vi khuẩn có hoạt tính hóa hướng hướng động âm đến clo (HTD 3.8, HTD 3.12 và HTD 3.15). Trong đó, HTD 3.8 thể hiện khả năng phản ứng tới chlorobenzene tốt nhất, với ngưỡng nồng độ khoảng 0.3 M. Sau khi thử khả năng hóa hướng động của HTD 3.8 với một số hợp chất chứa vòng thơm và/hoặc clo, chúng tôi nhận thấy HTD 3.8 phản ứng đặc hiệu cao với các hợp chất có chứa nhóm –C-Cl (bao gồm trichlomethane). Bên cạnh đó, điều kiện môi trường cho phản ứng hóa hướng động được tối ưu hóa thông qua nghiên cứu khả năng phản ứng của HTD 3.8 trong môi trường thạch bán lỏng với các yếu tố nhiệt độ, nồng độ NaCl và độ pH khác nhau. Dựa vào kết quả giải trình tự gene 16S rRNA, HTD 3.8 có trình tự tương đồng cao nhất với *Pseudomonas* sp. Những kết quả bước đầu nghiên cứu việc sử dụng trichloromethane như một phối tử cạnh tranh cho thấy HTD 3.8 có thể có một vài thụ thể hóa hướng động có khả năng cảm nhận và phát hiện các hợp chất có chứa liên kết –C-Cl.

Từ khóa: Hóa hướng động âm, chlorobenzene, hợp chất hữu cơ chứa clo.

Appendix

HTD 3.8 – 16S rRNA Gene Sequence

TGCTGCGTATGGATTCGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATAC
CGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCT
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CCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCC
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AAATTGCCGCTGTGAAAGAGATT