Study on Geosmin Degradation by Biofilm under Enhanced Organic and Inorganic Substrates Conditions

May 2013

Xiaowei TIAN

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A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Biotechnology

(Doctoral Program in Bioindustrial Sciences)

Xiaowei TIAN

Abstract

Geosmin, also known as *trans*-1, 10-dimethyl-*trans*-9-decalol, is a highly-odoriferous, earthy-smelling compound, produced by actinomycetes bacteria and cyanobacteria. In waterworks, geosmin is resistant to conventional water treatment such as coagulation and sedimentation. It is necessary to remove geosmin from water treatment plant. To date, efficient geosmin treatment methods include adsorption, oxidization and biodegradation. Although geosmin can be effectively removed by adsorption and oxidization, drawbacks are obvious, such as high cost, by-products, adsorbents regeneration required etc.. Based on this situation, biofiltration can be considered and selected because the mentioned drawbacks can be overcome in geosmin biodegradation.

Researches about geosmin degradation by biofiltration reported that geosmin degradation kinetic usually depended on the coexisting natural organic matter at mg L^{-1} in natural water. However, the effect of natural organic matter on geosmin degradation kinetic is uncertain when natural organic matter presented at μg L^{-1} in water treatment plant. Effect of carbon sources on geosmin biodegradation was studied in this thesis. Glucose, NaAc and HAC was added into culture to investigate geosmin biodegradation rate and mechanism. During geosmin degradation period, bacterial number, activity and community composition was analyzed by qPCR of 16S rDNA, ATP analysis and DGGE fingerprint, respectively. Biofilm was sampled in autumn. Glucose and sodium acetate stimulated geosmin degradation. Rapid geosmin degradation resulted from high bacterial activity under glucose and NaAc conditions.

Bacterial community composition severely changed during first two days under both conditions. Although HAC stimulated geosmin degradation within first day, geosmin degradation was repressed from the second day. During geosmin degradation period bacterial number increased, however, bacterial community composition did not change under HAC condition.

As well known, organic carbon source and inorganic nutrient substances are necessary for heterotrophic bacterial growth. Different nutrients may influence the population and composition of microorganism matrix in the biofilm. However, the effect of diverse nutrients on geosmin degradation and characteristics of microorganisms in biofilm have not been well-studied. In order to study effect of nutrient elements and dual nutrients on geosmin biodegradation, nitrate, ammonia and phosphate, glucose-nitrate, glucose-ammonia, HAC-nitrate and HAC-ammonia were selected as additives. The change of bacterial number, activity and community composition was investigated by DAPI stain method, ATP analyses and DGGE fingerprint. The winter biofilm was used in this study. Nitrate, ammonia and phosphate also stimulated geosmin degradation during incubation period through bacterial activity. Bacterial community composition also was influenced by nitrate, ammonia and phosphate. Geosmin degradation was stimulated by glucose-NO_3^- and glucose-NH_4^+ through bacterial activity. However, HAC-NO_3^- and HAC-NH_4^+ had no significant effect on geosmin degradation.

Geosmin biodegradation is a pseudo-first-order reaction and co-metabolic degradation in this study. Autumn biofilm had higher capability of geosmin

degradation than winter biofilm. Geosmin biodegradation was enhanced by carbon source and nutrient. Glucose had significant effect on geosmin degradation. HAC inhibited geosmin degradation because of low pH. Compared to nutrient, carbon source was main factor influence geosmin degradation.

Contents

Chapter 1 Introduction

1.1 Physical and chemical properties of geosmin

Geosmin, also known as *trans*-1, 10-dimethyl-*trans*-9-decalol, is a highly-odoriferous, earthy-smelling compound, isolated for the first time by Gerber and Lechevalier (1965) from cultures of actinomycetes bacteria (*Streptomyces* sp.), then identified by Gerber (1968) following chemical synthesis by Marshall and Hochstetler (1968). Geosmin is a chiral compound (Fig. 1.1), the (-) form is much more odoriferous than the (+) form (Darriet et al., 2001; Polak and Provasi, 1992). The (-) form is the one found in nature. The threshold of human is from 0.0001 to 0.015 μg L^{-1}, the natural occurring isomer (-) geosmin has an average 11 times lower threshold than (+) form (Persson, 1979; Polak and Provasi, 1992).

Geosmin, which is a bicyclic tertiary alcohol, has an approximate boiling point of 270°C - 271°C at 760 mm Hg and a vapor pressure of 5.49×10^{-5} atm, which make geosmin a semi volatile organic compound, and flash point of 104°C. Geosmin has a high $LogK_{ow}$ of 3.57 at 25°C and water solubility of 156.7 mg L^{-1} at 25°C which make geosmin being abstracted from water to hexane by solvent abstract (Pirbazari et al., 1992). Table 1.1 summarizes the basic properties of geosmin.

1.2 Geosmin producers

Odor outbreaks are caused by biological production of the naturally occurring (-) geosmin. Geosmin is produced by members of certain groups of benthic and pelagic

aquatic microorganisms found in aquatic environments such as lakes, reservoirs, and rivers. In addition, there are several other biological sources that are often overlooked, notably those which originate from terrestrial ecosystems, industrial waste treatment facilities, and drinking water treatment plants. Many of known producers are prokaryotes, which include both heterotroph and photoautotroph (Juttner and Watson, 2007). .

Production of geosmin has been documented for several different groups of heterotrophic microorganisms. In fact, this compound was originally identified from isolates of filamentous actinomycete bacteria (*Streptomyces* sp.) (Gerber, 1968; Medsker et al., 1968). The genus *Streptomyces* is widely used synonymously with odor-producing, but it is important to note that not all *Streptomyces* can produce geosmin, and nonstreptomyces such as *Nocardia* also can produce geosmin. Early actinomycete studies were highly influential, since they identified the structure and some of major biological sources of geosmin. Klausen et al. (2005) concluded that actinomycetes were responsible for low concentrations of geosmin in streams flowing past trout breeding aquaculture operations, because isolated strains of *Streptomyces* from the habitats were able to synthesize geosmin. But a careful review of geosmin literature to date reveals that actinomycetes have been clearly implicated in comparatively few episodes (Gerber and Lechevalier, 1965; Tung et al., 2006).

Cyanobacteria are considered to be the major sources of geosmin in aquatic environments where photosynthetic growth is possible (Jüttner et al., 2008; Matsumoto and Tsuciya, 1988). More than 200 studies have made considerable

advances in our knowledge of the biochemistry, taxonomy and ecology of some of the cyanobacteria which produce geosmin (Komárek, 2010; Matsumoto and Tsuchiya, 1988; Whitton, 1992). Hindák (2000) discussed main generic diagnostic characters of *Aphanizomenon* and *Anabaena.* The shape and size of akinetes in *Aphanizomenon* and *Anabaena* are so conspicuously different that they can be classified as two independent but closely related taxa. Cyanobacteria were known as geosmin producers (Medsker et al., 1968; Safferman et al., 1967), but it was not recognized as acute geosmin producer until publishing the important study by Tabachek and Yurkowski (1976).

Trowitzsch et al. (2006) first reported that myxobacteria produce the geosmin and great abundance of myxobacteria in soils all over the world. So this organism must be regarded as another important source of geosmin in water environments. Mattheis and Roberts (1992) first reported that fungi produce the geosmin.

1.3 Geosmin induced problems

Geosmin is one of a few chemicals that are responsible for the characteristic earthy smell of soil (Gerber, 1979). Although geosmin has some value in formulating fragrances (Eaton and Sandusky, 2010) and as a natural component of some foods (Maga, 1987), in many circumstances its presence is not desirable such as when it occurs in wine (Prat et al., 2008), aquaculture (Smith, 1988), and drinking water (Zaitlin and Watson, 2006).

Geosmin was identified in wine, Chinese liquor and juice by GC-olfactometry

analysis, and its presence was due to contamination by contaminating microorganisms (Amon and Simpson, 1986; Amon et al., 1989). In aquaculture, fish uptake and accumulate geosmin in flesh, and subsequent rejection of fish by processors (Shelby et al. 2004). These geosmin episodes force producers to hold the crop until the catfish are purged of the compounds and are determined to be odorless. This additional holding period causes economic losses to producers due to the additional expense of feed, work time, water treatment chemicals and harvesting time problems. In addition, delayed harvest can result in the loss of catfish to disease, poor water quality and bird depredation. Estimated annual economic losses are from $15 to $23 million for catfish producers (Shelby et al., 2004).

The presence of taste and odor in potable water supplies is an increasingly frequent problem. These problems are recognized (Table 1.2). Some of them will be discussed in more detail below. Burlingame et al. (1986) documented that two T&O incidents occurred in the Schuylkill River in Philadelphia, and effective eradication of odor in both cases was a result of an existing T&O control program. The Yodo River debouches from Lake Biwa (Japan) have experienced earthy odor problems, but geosmin concentration decreased along the course of a river because of biodegradation (Hishida et al., 1988). Diamond Valley Lake, a large new reservoir in southern California, had T&O problems in almost 6 years since 1999.

Earthy odor in water is a common source of customer complaints for water utilities. A survey conducted in more than 800 utilities in United States and Canada had found that 16% of the utilities experienced serious earthy problems, and that utilities spend

an average of about 4.5% of their total treatment budget on earthy control. Control and removal of earthy odors is a worldwide concern.

Problems associated with T&O contamination in drinking water give rise to derogations of consumer confidence, consumer satisfaction and water consumption. Tap water with detectable T&O may be perceived by the consumer as unsafe to drink even though it adapts to the guideline for regulated constituents. Although neither the Unite States Environmental Protection Agency (USEPA) nor the World Health Organization (WHO) has declared geosmin as a health hazard, geosmin can lead to acute health effects such as heat exhaustion and sunstroke, or chronic health effects such as kidney problems (Simpson, 2008).

Surface waters are an important source for drinking water throughout the world. Some of the major cities in the world depend on filtration to obtain their drinking water from a large extent on reservoirs, natural lakes or river bank. Normally, surface water has to be treated in a multi-step procedure consisting of particle separation, oxidation and adsorption to fulfill the requirements with respect to microbiological quality, toxic compounds and aesthetic aspects such as T&O problem. Despite this extensive water treatment, many water utilities are confronted with T&O complaints (Suffet et al., 1996). Conventional water treatment plants achieve minimal removal of geosmin, which concentration is higher than commonly accepted organoleptic detection level by human noise (5 to 10 ng L^{-1}) (Yong et al., 1996). Therefore, water utilities are anxious to quickly and efficiently mitigate T&O problems (Peter et al., 2009).

1.4 Geosmin removal methods

As former mentioned, geosmin removal method is required. Because of physico-chemical properties of geosmin, geosmin can be removed in the treatment process including oxidation, adsorption, biodegradation and radiation (Table 1.3). Every method possess advantage and disadvantage, the details are as followed.

1.4.1 Oxidation

Oxidation is the major chemical treatment process for T&O removal. Oxidizing agents utilized in the water industry include ozone (O_3), hydrogen peroxide (H_2O_2) and ultraviolet (UV). Ozone is one of the most efficient agents for T&O removal. There are many studies for ozonation of geosmin. For example, Lalezary et al. (1986) found that O_3 (up to 8 mg L^{-1}) achieved less than 30% destruction of geosmin in organic free water. Terashima (1988) documented that doses between 2 and 5 mg L^{-1} of O_3 led to 7-100% destruction of geosmin in natural water.

Intermediate reaction products are formed during water treatment with ozone. The type and quantity of these disinfection by-products depends upon ozone dosage, reaction time, radical inhibiting agents/scavengers and pH. Aliphatic and aromatic aldehydes formation (>C6), is frequently reported in the literatures (Anselme et al., 1988; Schalekamp, 1983). Ozone can oxidize bromide to hypobromous acid, which then reacts with organic matter to form brominated by-products, these products are harmful to human (Koch et al., 1992).

Ozone oxidization process in water treatment involves hydroxyl radicals as

intermediate which is depends on natural organic matter (NOM). UV/H_2O_2 provides oxidation through generation of hydroxyl radicals by photolysis of H_2O_2. This process degrades recalcitrant odorants geosmin mainly by the reaction with hydroxyl radicals. UV and H_2O_2 combination will be used as one of the major tools in the fight against chronic T&O (Rosenfeldt and Linden, 2007). Collivignarelli and Sorlini (2004) reported that O_3/UV was more efficiency and complete remove geosmin than ozone in natural water. Reduction of bromated was also significant for O_3/UV.

Ozone, H_2O_2 and UV can complete remove geosmin in natural water, although doses are higher than that required for disinfection, which cause increasing financial budge for water utilities. In addition, addition of chemicals is expensive and can result in formation of disinfection by-products (DBPs), which are unacceptable due to health and regulatory concerns.

1.4.2 Adsorption

As disadvantage of oxidation, adsorption is attracted for water utilities. Adsorption with powdered activated carbon (PAC) or granular activated carbon (GAC) is being widely used in drinking water treatment plants, mainly for removing organic pollutants.

Various studies have looked into use of activated carbon for geosmin remediation (Crozes et al., 1999; Drikas et al., 2009; Srinivasan and Sorial, 2011). Ridal et al. (2001) investigated the long-term performance of GAC filter beds in a water treatment plant in Canada. Geosmin was removed to at or below the threshold

concentration (less than 10 ng L^{-1}) after two months operation. While the performance of the GAC filters was monitored 1 and 2 year later, it had dropped significantly and the effluent geosmin concentrations were higher than the threshold concentration. The authors suggested that GAC was coated and mixed with dissolved organic carbon (DOC) and then removal performance decreased. It is likely that competitive adsorption was a bigger factor to reduce the performance than some of the factors pointed out by the authors.

As seen from the above discussion, the NOM is the main factor effect the successful application of GAC/PAC adsorption for geosmin removal. So prior to application GAC/PAC, it is important to understand its adsorption characteristics in the presence of NOM, which is commonly found in water.

1.4.3 Radiation and others

Vajdic (1971) found that the treatment with gamma rays was very effective for removing musty and earthy flavors. He found that the efficiency is very high, but the cost is very expensive. Also, some by-products such as nitrite ions will make the water undrinkable (Montiel. 1983).

Ultrasonic irradiation and nanofiltration are treatment methods that have shown promise for removing geosmin and MIB. Song and O'Shea (2007) reported that ultrasonic irradiation at 640 kHz provided 90% removal of both geosmin and MIB within 30 minutes. Several researchers had demonstrated that nanofiltration membranes are effective at removing earthy/musty odors (Choi et al., 2010; Dixon et

al., 2010). While both of these methods have shown potential for geosmin removal, at the present moment, they are expensive.

1.4.4 Biodegradation

Alternatively, geosmin is susceptible to biological degradation, having implicated a variety of microorganisms (Table 1.4). The biodegradation susceptibility of geosmin can be attributed to their structures which is similar to biodegradable alicyclic alcohols and ketones (Rittmann et al., 1995; Trudgill, 1984)

Hoefel et al. (2006) identified three gram-negative bacteria from water treatment plant sand filter that coordinated biological degradation of geosmin, and interestingly the degradation did not occur even if one of the three isolates was absent. Hoefel et al. (2009) firstly reported that gram-negative bacterium Geo48 has the ability of degrading geosmin individually. Following on Hoefel et al. (2009), Zhou et al. (2011) isolated three strains of gram-negative bacteria which had capable of removing geosmin from drinking water.

As the aforementioned, bacteria which can degrade geosmin were isolated from biofilms. It indicates that there is a potential for removing T&O compounds by using biological filtration processes. Biological filtration systems for removing organic contaminants are becoming more attractive to water suppliers, as they are generally low technology, requiring little maintenance and infrastructure. Furthermore, such systems are able to remove the contaminants without addition of other chemicals, which may have the potential to produce by-products in drinking water.

In the last decade, there has been an extensive use of biological treatment for removing contaminants in water and wastewater. Unlike wastewater, biological methods have been limited for applying in drinking water. Biological methods are mainly used with filtration, or biofiltration. Huck et al. (1995) were one of the first researchers to study biological removal of odor causing compounds in drinking water. They studied microbial geosmin removal using a lab scale bioreactor, and the results demonstrated very low geosmin removal. They concluded that biodegradation was not the most effective technology for removing T&O compounds in drinking water.

However subsequent researches have shown contrary results. For example, Ho et al. (2007) reported geosmin removal by a biologically active sand filter. They used river water which was known about significant odor outbreaks due to geosmin. However, the treated water through a treatment plant (sand filter) was surprisingly free of any geosmin. This result corroborated the removal of geosmin by biological sand filtration. The authors also determined the pseudo-first-order reaction kinetics by running batch experiments with the biofilm obtained from one of the sand filters with rates as high as 0.6 d^{-1}. Jüttner et al. (2008) reported that a slow sand filtration unit (flow rate of 420 L m^{-2} d^{-1}) achieved excellent rates of geosmin elimination.

However, currently, studies regarding the biodegradation of MIB and geosmin are limited. Westerhoff et al. (2005) conducted batch incubation using lake water and they modeled MIB and geosmin biodegradation as a pseudo-zero-order reaction. In contrast, Rittmann et al. (1995) determined that MIB and geosmin would be utilized as secondary substrates in natural water, due to the presence of NOM which is present

at much higher concentrations than T&O compounds. Consequently, they determined the biodegradation of MIB and geosmin in natural water to be a second-order reaction.

Meanwhile, no definitive pathway has been elucidated for the biodegradation of geosmin. Saito et al. (1999) identified four possible biodegradation products of geosmin, and two of which were identified as 1,4a-dimethyl- 2,3,4,4a,5,6,7,8 -octahydronaphthalene and enone. Interestingly, these biodegradation products also used in the chemical synthesis of (-)-geosmin (Saito et al., 1996).

Trudgill (1984) documented that strains of *Acinetobacter* and *Nocardia* were capable of degrading cyclohexanol via monooxygenase enzymes, similar to the biological Baeyer-Villiger reaction. Cyclohexanol is initially oxidized to an alicyclic ketone. The insertion of a ring oxygen atom follows via a monooxygenase enzyme. The resultant lactone is unstable, causing the lactone to be hydrolyzed into a diacid. It is possible that geosmin may be biodegraded by a pathway similar to that of cyclohexanol. Up to date, geosmin biodegradation pathway is unclear, and geosmin biodegradation was affected by NOM. So I speculate that geosmin biodegradation pathway was affected by NOM. If geosmin biodegradation pathway is affected by NOM and geosmin biodegradation pathway is the same as cyclohexanol, organic carbon source which induce acidic condition will inhibit geosmin biodegradation. The final production is easy to hydrolysis into a diacid, and this step might be inhibited by acidic carbon sources.

1.5 Objectives

It takes more than 40 years to find out the geosmin biodegradation by bacterium. However, geosmin biodegradation mechanism and pathway have not been identified, which may be result from geosmin can't be utilized as sole carbon source for bacterium. Looking back to geosmin biodegradation studies, geosmin degradation by bacteria were almost done when NOM presented as high concentration. So why geosmin was only degraded under NOM present condition? Was all these processes can be explained as co-metabolism? To date, Saito et al. (1999) reported geosmin degradation was stimulated by adding ethanol. Were this condition really widespread and the only way of geosmin degradation by biofilm?

This thesis aimed to study geosmin degradation mechanism by biofilm when the low concentration of 1) organic carbon source (glucose, sodium acetate and acetic acid), 2) inorganic nutrient substance (nitrate, ammonia and phosphate), and 3) dual nutrients substrates (glucose-nitrate, glucose-ammonia, acetic acid-nitrate, and acetic acid-ammonia) coexist with geosmin. I try to find powerful evidence to confirm that geosmin degradation is co-metabolism, as Saito et al. (1999) adding ethanol to enhance geosmin degradation based on co-metabolism conception.

1.6 Thesis contents

In this thesis, my objectives were to investigate carbon source and nutrient effect on geosmin biodegradation, and compare effect of carbon source and nutrient on geosmin biodegradation. To achieve these purposes, the following studies were

carried out.

In chapter two, organic carbon sources were added into culture, including glucose, sodium acetate and acetic acid. Biofilm was obtained from the water treatment plant in September 2012. For insight into the effect of carbon source, the qPCR of 16S rDNA, ATP analysis and DGGE fingerprint was used to investigate bacteria number, activity and community composition. Geosmin was monitored by GC/MS.

In chapter three, nitrate, ammonia and phosphate were added into culture to investigate inorganic nutrient effect on geosmin biodegradation. Dual nutrient substance co-action effect on geosmin degradation was also done in this chapter, the additives including glucose-nitrate, glucose-ammonia, HAC-nitrate and HAC-ammonia. Biofilm was sampled from the same place as chapter two in December in 2012. DAPI stain method, ATP analysis and DGGE fingerprint was used to study bacterial number, activity and community composition, which will uncover reason of geosmin degradation.

In chapter four, conclusions were drawn and the future plans about geosmin pathway were made.

Table 1.1 Physico-chemical properties of geosmin

Formula	$C_{12}H_{22}O$
Composition	C (79.06%), H (12.16%), O (8.78%)
Molecular weight	182.31
Henry's law constant [a]	6.66×10^{-5} atm m^3 mol^{-1}
Density [a]	0.9494 g cm^{-3}
Refractive Indices [a]	1.4650
Boiling point (760 mmHg) [b]	270-271°C
Vapor pressure [a]	5.49×10^{-5} atm
Flash point [b]	104°C
$LogK_{ow}$ (25°C) [b]	3.57

[a] Pirbazari et al. (1992)

[b] Ultimate survey model (USEPA 2009)

Table 1.2 Problems of geosmin induced in drinking water

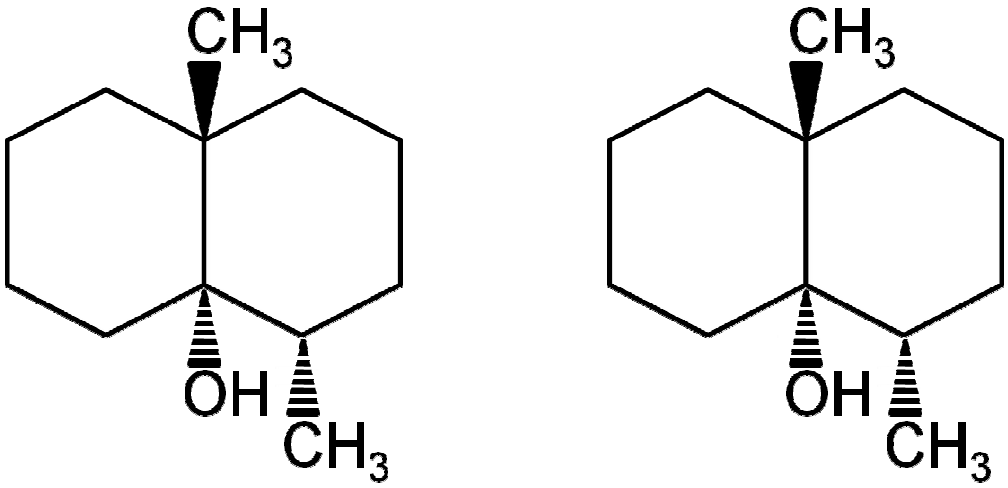
Country		Reference
USA	Schuylikill River	Burlingame et al. (1986)
Canada	Buffalo Pound Lake	Slater and Block (1983)
Japan	Yodo River basin	Yagi et al. (1983)
	Nunobiki reservoir	Yano et al. (1988)
	Water source of Tokyo	Matsumoto and Tsuchiya (1988)
Norway	Lake Mjosa and River Glama	Berglind et al. (1983)
Israel	Tsalmon reservoir	Leventer and Eren (1969)
Australia	Water source	Hayes and Burch (1989)
South Africa	Nagle Dam	Wnorowski (1992)
Taiwan	Fengshen reservior	Tung et al. (2008)
Switzerland	Lake Zürich	Durrer et al. (1999)

Table 1.3 Geosmin removal treatments

Treatment		
Chemical methods	Physical methods	Microbial methods
Chlorine	Aeration	Single-strain bacterium
Chloramines	Powered activated carbon	Biofilm
Chlorine dioxide	(PAC)	Yeasts
Ozone	Granular Activated carbon	Protozoa
Permanganate	(GAC)	
	Dissolved air flotation (DAF)	
	Radiation	
	Gamma rays	
	UV light	
	High energy	
	Electrons	

Table 1.4 List of geosmin degradation microorganisms

Microorganisms	Isolation source	Reference
Bacillus cereus *Sphingopyxis alaskensis*	biologically active sand filters	Ho et al. (2007)
Bacillus subtilis	bio-activated carbon filter	Yagi et al. (1988)
Arthrobacter atrocyaneus	biologically active sand filters	Hoefel et al. (2009)
Chryseobacterium sp. *Sinorhizobium* sp. *Stenotrophomonas* sp.	biologically active carbon	Zhou et al. (2011)



(+)-geosmin (-)-geosmin

Fig. 1.1 Molecular structural formula of (+)-geosmin and (-)-geosmin

Chapter 2 Effect of glucose, sodium acetate and acetic acid on gesomin degradation by biofilm

2.1 Introduction

Geosmin biodegradation by biofilm is proposed as a major pathway for natural elimination of geosmin (Juttner and Watson, 2007). Microorganisms always accumulate in polysaccharide matrices and form structural and functional microbial assemblages on submerged surfaces that are commonly known as biofilm (Grützmacher et al., 2002). Naturally-originated biofilm affect the fate of water contaminants through their adsorption and biodegradation capacities (Pusch et al., 1998).

Westerhoff et al. (2005) conducted batch culture experiments of geosmin biodegradation using lake water, and they imitated geosmin degradation as a pseudo-zero-order reaction. In contrast, Rittmann et al. (1995) determined geosmin biodegradation in natural water to be a second-order reaction, due to the presence of NOM which was present at much higher concentration than geosmin (mg L^{-1}, compared with ng L^{-1}). Ho et al. (2007) reported that geosmin biodegradation was a pseudo-zero-order reaction. Saito et al. (1999) identified four possible biodegradation products of geosmin, two of which were identified as 1,4a-dimethyl-2,3,4,4a,5,6,7,8-octahydronaphthalene. The most important result of this study was the addition of ethanol to enhance geosmin biodegradation. This based on co-metabolism concept.

Geosmin biodegradation was influenced by NOM. In order to remove geosmin from water which contain NOM at μg L^{-1}, water treatment plant can adopt biofilter method. But NOM influence for geosmin biodegradation in biofilter step is not clear; especially NOM concentration in water is not high, as μg L^{-1} level.

This chapter aims to identify geosmin biodegradation mechanism by biofilm when different organic carbon source (at μg L^{-1}) was coexisted with geosmin. Glucose, sodium acetate and acetic acid is selected and used for organic carbon source, and naturally-originated biofilm from a water treatment plant as a inocula, and the change of bacterial number, activity and community compositions in the biofilm were monitored by 16S rDNA copy number, ATP and DGGE fingerprint.

2.2 Materials and methods

2.2.1 Chemical

Geosmin standard material was purchased from WAKO pure Chemicals Ltd. Osaka, Japan. The authentic sample of 20 mg was dissolved in ultra pure water (Resistivity 18.0 MΩ·cm at 25°C). The stock solution was transferred into brown air-tight glass bottle and stored in the dark at 4°C prior to use.

2.2.2 Batch biodegradation experiments

Lake Kasumigaura (Japan) serves as a water source for many cities in Ibaraki prefecture. The nearby waterworks Southern Ibaraki Prefectural Waterworks utilizes the biological treatment facilities. Unit packed with a honeycomb tube is used as

carrier for biofilm habitat in these biological treatment facilities. The maximum water supply quantity is 160,000 m^3 d^{-1} and hydraulic time of 2 h. Honeycomb tube is made by polyvinyl chloride with a thickness of 0.1 mm. Biofilm was scraped from the honeycomb tube as inocula in September 2012. The honeycomb was taken out from water and using sterile ladle to scraped biofilm attached at the surface of honeycomb tube. Biofilm was collected in sterile centrifuge tube (15 mL) which was stored at 4°C ice box before use.

Geosmin biodegradation experiments were conducted in 1000 mL conical flasks. In each flask, biofilm (fresh, 1.0 g) was added to 500 mL sterile mineral salt medium (MSM) (pH=7.6) including (per liter of ultra-pure water) 210 mg $CaCl_2 \cdot 12H_2O$, 130 mg $MgSO_4 \cdot 7H_2O$, 50 mg $Na_2HPO_4 \cdot 12H_2O$, 10 mg K_2HPO_4, and 20 mg $(NH_4)_2SO_4$. The culture was spiked with geosmin to establish an initial concentration 500 ng L^{-1}. As 400 μg L^{-1} and 300 μg L^{-1} TOC of glucose and sodium acetate (NaAc) was spiked into culture, respectively, which is lower than NOM concentration level (mg L^{-1}) occurring in Lake Kasumigaura. Acetic acid (HAC) was added to form concentration of 0.01% V/V. HAC was added base on hypothesis that geosmin degradation pathway was same as cyclohexanol, final product of which was unstable diacid, and dosage was reduced because of pH value. The control was conducted which contained sterile biofilm (autoclaved at 121°C for 20 min) and geosmin to investigate abiotic impact. The subsample of 15 mL was took from each flasks at 0, 1^{st}, 2^{nd}, 4^{th}, 6^{th}, 8^{th} day for analysis.

2.2.3 Geosmin analysis

Hexane extraction analysis for geosmin and MIB (Jensen et al., 1994) was used to extract the geosmin. Progress of geosmin biodegradation in each flask was monitored by GC/MS analysis in a 2010 plus GC/MS (SHIMADZU, Japan) equipped with a RESTEK Rix–5MS capillary column (30 m x 0.25 mm ID x 0.25 μm fixed phase) with helium as carrier gas. GC-MS condition was list in Table 2.1.

2.2.4 TaqMan qPCR analysis

For extraction of bacterial DNA, 5 mL of subsample was filtered onto 0.2 μm membrane filter (Nuclepore Track-Etch Membrane, Whatman, UK). The filter was put into the 1.5 mL Eppendorf tube adding 180 μL lysis buffer (50 mM Tris (pH=8.3); 40 mM EDTA; 0.75 M sucrose) to break the cell wall then stored at -80°C before extraction. DNA extraction was based on phenol–chloroform extraction method (Ch omczynski and Sacchi, 1987. Firstly, adding 5 μL of 50 mg mL^{-1} lysozyme and incubated for 1 h at 37°C. Then 5 μL of 20 mg mL^{-1} proteinase K and 20 μL of 10% SDS was added to break the wall of bacteria, and the mixture was incubated for 2 h at 55°C, followed by addition of the same volume PCIA (phenol: chloroform: isoamyl alcohol, 25:24:1, pH=8.0), gently turned tube over for several times followed by centrifugation (15000 g, 30 min, 25°C) and removal of the supernatant for next abstraction. PCIA abstraction had done for 1-2 times. After PCIA abstraction, the same volume CIA (chloroform: isoamyl alcohol, 24: 1) was added, and then centrifugation (15000 g, 30 min, 25°C), taking the supernatant to the new tube. Then

added one-tenth volume of the upper layer of sodium acetate (3 M, pH=7.5) and 2.5 times volume of supernatant of ethanol (100%) to the tube, freezed at -30°C over night. Centrifuging (15000 g, 30 min, 4°C) and discarding supernatant and using 70% ethanol 1 mL to wash DNA and then removing all ethanol and dry up. Then DNA dissolved in TE buffer (pH=8.0) stored at -30°C for use.

The bacterial number was quantified by quantitative polymerase chain reaction (qPCR) using BACT1369F/PROK1492R primer set and TM1389BACT2 probe (Table 2.1) (Suzuki et al., 2000). DNA standard curve for total bacterial number was prepared from a serial dilution of purified 1465 bp bacterial 16S rDNA fragment, from the strain *Sphingomonas* sp. MD-1 (Saitou et al., 2003). Results of the 16S rDNA were linear between 2.67×10^{0} and 2.67×10^{6} copies μL^{-1} with R^2 values of 0.99. qPCR were performed in triplicate and each 20 μL of reaction mixture containing primer 0.3 μM and probe 0.2 μM at final concentration, 10 μL of THUNDERBIRD Probe qPCR Mix, 0.04 μL of 50×ROX reference dye (Tyoko Co. Ltd., Osaka, Japan) and 1.0 μL of each DNA standard or sample template. Thermal profile consisted of an initial denaturation at 95°C for 1 min followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 56°C for 45 s. The increase in fluorescent signal was measured at each annealing step. The real-time PCR was performed using a 7500 real-time PCR system (Applied Biosystems, Foster City, USA).

2.2.5 ATP analysis

The ATP standard curve was prepared from a serial dilution of standard ATP

solution, results of linear between 1 μM to 10 pM. Mixing 100 μL BacTiter-Glo^{TM} Reagent (Promega Corp., Madison, WI) and 100 μL water subsamples or standard in each hole of an opaque-walled multiwall plate and incubated for 1 min to insure no lysis required to release ATP. Then luminescence was recorded by Fluoroskan Ascent type 374 multiwell plate reader (Labsystems, Helsinki, Finland).

2.2.6 PCR-DGGE analysis

16S rDNA fragment of bacteria was amplified by PCR using the DGGE universal primer set 968F-GC/1401R (Table 2.2) (Valáškováand Baldrian, 2009). The reaction mixture was 20 μL containing as, 1 μL of template DNA, 0.5 μL of each primer (20 μM), 2 μL of 10×PCR buffer for Ex Taq (20 mM Mg^{2+} plus), 1.6 μL of dNTP mixture, and 0.25 μL of Ex Taq DNA polymerase (TAKARA BIO INC. Japan). PCR was performed with a Veriti 200 PCR Thermal Cycler (Applied Biosystems, Foster city, USA). The thermal profile for amplification was: 5 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C; and final 7 min at 72°C.

Products of PCR reaction were analyzed by subsequent DGGE based on the protocol of Muyzer et al. (1993). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1×TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH=7.4). To separate the amplified PCR fragments, the polyacrylamide gels were made with denaturing gradients ranging from 45% to 55%. On each gel, a DGGE marker (DGGE Marker **I**, NIPPON GENE, Japan) was loaded, which was required for processing and comparing the different gels. The electrophoresis was run for 12.5 h at 60°C at a

constant voltage of 80 V, which was performed by D-Code system (Bio-Rad, Hercules, CA). Following electrophoresis, the gel was stained for 1.0 h in Nucleic Acid Gel Stain (Gel-Red, Biotium, Hayward, CA) solution in 1×TAE.

The obtained DGGE fingerprint were subsequently normalized and analyzed with the Quantity One software version 3.1 (Toyobo, Tokyo, Japan). During this processing, the different lanes were defined, background was subtracted, differences in the intensity of the lanes were compensated during normalization, and the relative quantity was calculated, and the data were analyzed for PCA. A covariance data was extracted with pairwise deletion and varimax factor rotation. Data reduction provided a two-factorial ordering of the variance of DGGE profiles, which was plotted as a schematic diagram.

2.2.7 Statistics analysis

Geosmin biodegradation rate was calculated by linear regression of geosmin concentration of the natural logarithm remained in culture as function of time. For "only geosmin" regression of concentration as function of 4 days, meanwhile, for glucose and NaAc was 2 days. For HAC the rate was calculated within 1 day.

A one-way ANOV and principal components in the statistical Package for Social Science v17.0 software (SPSS Inc IL, USA) was applied to determine if there were significant differences between carbon sources and analyze principal components of DGGE fingerprint. Statistical significance was accepted at $p<0.05$.

Simple mathematical index was calculated for each fingerprint arising from

PCR-DGGE analysis of the samples from only geosmin, glucose, NaAc and HAC. The bands considered in the analysis were the ones automatically and manually detected and by the software Quantity One. Indexes of biodiversity of every sample were calculated as following:

$$IB = {}^{n}/_{n_M} \quad (2\text{-}1)$$

The biodiversity index *IB* was meant to express the degree of microbial complexity for each sample. Where n is the number of DGGE bands in the profile; n_M is the number of bands counted in DGGE profile with the maximum number of bands. The number of bands detected in a DGGE lane was used as a measure of the number of species presence, the relative quantity was used as species richness.

2.3 Results and discussion

2.3.1 Geosmin degradation under various carbon source conditions

Figure 2.1 shows the time course of geosmin concentration change with natural biofilm in the presence of several organic carbon sources. Reduction of geosmin was observed in control which was attributed to abiotic impact, like volatilization and adsorption. For only geosmin (biofilm with geosmin), there was no obvious lag phase and geosmin was gradually degraded within 4 days. The final geosmin removal was 99% (including abiotic loss) in only geosmin condition, which was agreed with the study of Xue et al. (2012) that final geosmin removal was more than 90% in most months of the year. This result indicated indigenous bacteria in the biofilm still had ability to degrade geosmin; even geosmin concentration in influent water at water

treatment plant was negligible. Geosmin biodegradation rate constant (K) was 0.55 d^{-1} (R^2=0.95), which was a pseudo-first-order reaction, consistent with the study of Ho et al. (2007) that geosmin biodegradation rate constants were between 0.1 d^{-1} and 0.58 d^{-1} in treated Morgan water treatment plant water.

Glucose and NaAc had same final geosmin removal efficiency (99%) as only geosmin (Fig. 2.1). There was no lag phase in the case of adding glucose and NaAc. Once experiment started, geosmin was rapidly degraded to below 10 ng L^{-1} within 2 days. Figure 2.1 showed the same decrease pattern of geosmin concentration under glucose and NaAc condition, which meant NaAc and glucose stimulated geosmin degradation. Geosmin degradation rate constant (K) were 1.35 d^{-1} (R^2=0.90) and 1.19 d^{-1} (R^2=0.92) for glucose and NaAc, respectively. These results were consistent with Saito et al. (1999) who documented geosmin degradation was accelerated through adding ethanol based on the concept of co-metabolism. Co-metabolism is defined as transformation of a non-growth substrate by growing cells in the present of a growth substrate, by resting cells in the absence of a growth substrate, or by resting cells in the presence of an energy substrate (Criddle, 2004). I suspected that co-metabolic enzymes and cofactors were induced by glucose and NaAc under geosmin biodegradation

HAC resulted in evident inhibition of geosmin biodegradation, compared with only geosmin condition (Fig. 2.1). Final geosmin removal was just 59%, which indicated that geosmin biodegradation was repressed by HAC. However, geosmin biodegradation rate constant was 0.52 d^{-1} (R^2=0.99) in the first day, which suggested

geosmin was degraded in the first day, but immediately repressed. This result might be an evidence for hypothesis that geosmin was degraded by a pathway similar to that of cyclohexanol. Trudgill (1984) documented that strains of *Acinetobacter* and *Nocardia* were capable of degrading cyclohexanol via monooxygenase enzymes, similar to the biological Baeyer-Villiger reaction (ten Brink et al., 2004). Cyclohexanol was initially oxidized to an alicyclic ketone. The insertion of a ring oxygen atom followed via a monooxygenase enzyme. The resultant lactone was unstable, causing the lactone to be hydrolyzed into a diacid. The first day geosmin was degraded and some factors held up geosmin degradation from the second day under HAC condition. So it is speculated that HAC or accumulated lactone caused some damages to geosmin biodegradation.

Glucose had significant stimulation effect on geosmin degradation compared with other carbon sources. It seems to connect glucose degradation pathway with geosmin biodegradation. Bacteria utilizing one carbon atom can produce 4 ATP for glucose and acetate. However, glucose should provide more energy than acetate because glucose contains more C-C bonds, and less energy is required for the synthesis of cell material (Paul et al., 1989). So the glucose would have significant stimulation effect on geosmin degradation.

2.3.2 Bacterial number and activity change during geosmin degradation

16S rDNA copy number of only geosmin condition increased from the 0 to 2nd day, and then gradually decreased during incubation period (Fig. 2.2). Change of ATP

concentration had a clear correlation with geosmin concentration under only geosmin condition (Fig. 2.3). Correlation coefficient (R^2) of geosmin concentration and ATP concentration was 0.93 (p<0.05). Correlation coefficient (R^2) of geosmin concentration and 16S rDNA copy number was 0.83 (p<0.01). These indicated that geosmin in only geosmin condition was not degraded as growth substrate and energy substrate. Some uncharacterized carbon might induce geosmin biodegradation because of slight fluctuation of bacterial number (Fig. 2.2). These carbons might be the excretion of bacteria.

16S rDNA copy number of glucose and NaAc condition was not changed dramatically during the experiment (Fig. 2.2). On the other hand, glucose and NaAc had the same effect on ATP concentration (Fig. 2.3), which stimulated bacterial activity within 2 days and the highest level were 201 pmol mL^{-1} and 134 pmol mL^{-1} of glucose and NaAc, respectively. It could be concluded that this two carbon sources stimulated bacterial activity, and rapid geosmin biodegradation was attributed to this stimulation. On the 2nd day geosmin concentration reached to less than 10 ng L^{-1} under both condition. When bacterial activity decreased from the 2nd day, geosmin biodegradation decreased. Compared to 16S rDNA copy number, glucose and NaAc were likely to just stimulate ATP increase, which indicated that glucose and NaAc were utilized by bacteria as energy substrates. An energy substrate is defined as an electron donor that provides reducing power and energy, but does not by itself support growth, while a growth substrate is defined as an electron donor that provides reducing power and energy for cell growth and maintenance (Criddle, 2004).

Geosmin biodegradation was induced by bacteria utilizing glucose and NaAc as energy substrate, because bacterial number did not increase under these two conditions. This phenomenon proved that geosmin biodegradation was co-metabolic biodegradation by bacteria. Studies about geosmin biodegradation were done with two kinds of water, one was water treatment plant water (Ho et al., 2007; Hoefel et al., 2006; Hoefel et al., 2009; McDowall et al., 2009; Zhou et al., 2011) and the other was natural water Ho et al., 2012a; Ho et al., 2012b), which contain certain organic carbon. The DOC concentrations were from 2.0 mg L^{-1} and 12.8 mg L^{-1}, and the geosmin degradation rate constant was between 0.098 d^{-1} and 0.696 d^{-1} in these studies. These high DOC concentrations would induce geosmin degradation by bacteria. Zhou et al. (2011) also reported that geosmin was also degraded when geosmin concentration was 2 mg L^{-1} in mineral salt medium. However, the rate constant was 0.097 d^{-1}. These results showed that geosmin degradation was enhanced by DOC.

Figure 2.2 shows that 16S rDNA copy number of HAC condition slightly fluctuated in first two days, but in the next day bacterial number increased until the end of experiment. Interestingly, geosmin concentration almost did not change when 16S rDNA copy number increased from the 2nd day. This interesting phenomenon indicated that the indigenous bacteria had ability of utilizing geosmin, but after one day, some by-products or factors produced by HAC utilization might inhibit geosmin biodegradation. This factor might be acid, because ATP concentration could not be detectable from the 2nd day in HAC condition. Compared with only geosmin condition, HAC stimulated bacterial growth (Fig. 2.2), and this result further proved

hypothesis that geosmin biodegradation pathway was the same as cyclohexanol, which was degraded to hydrolysable diacid.

2.3.3 DGGE fingerprint

Figure 2.4 showed DGGE fingerprints during the geosmin degradation experiment in the presence of several organic carbon sources. One band in DGGE fingerprint represents one genus bacteria in theory (Muyzer and Smalla, 1998). Figure 2.4 showed that although dominant bacteria appeared under all conditions, the dominant bacteria was different from each other. In all condition, bacterial diversity decreased by the end of incubation, which proved that carbon sources regulated the bacterial diversities.

The bacterial diversity *IB* value in initial day of only geosmin was 0.43 and gradually decreased to 0.28 by the end of the experiment, which implied that during geosmin degradation dominant species changed and bacterial diversity decreased. For glucose, the *IB* value of first day was 0.28 and decreased to 0.15 on the eighth day. These results showed that when glucose was added in culture, bacterial species changed and the bacterial diversity decreased. For NaAc, the IB increased from 0.15 to 0.29 during incubation which implied that bacteria utilizing NaAc, and the bands (H, I, J, and K) position of NaAc conditions was different from only geosmin's band position. For HAC, the *IB* value decreased from 0.28 to 0.12. The lane profile final of day 10 was different from other three conditions.

Figure 2.5 shows the PCA analysis using DGGE fingerprints. Significant difference

of microbial community compositions were observed between “only geosmin” and additional carbon sources condition. The PCA plots of “only geosmin” were mainly located at right area, while the plots of glucose, NaAc and HAC were located at left area in the figure. It is indicated that additional carbon sources had significant effects on changing bacterial community compositions during geosmin biodegradation period. Additional carbon sources obviously induced community structures change, depending on carbon sources.

Various environment factors regulated bacterial community compositions such as pH, carbon source species and concentration of bacterial metabolites (Singh et al. 2006). The complexity level of bacterial diversities was as following: “only geosmin” > glucose and NaAc > HAC (data not shown). Consequently, it can be concluded that additional carbon sources such as glucose, NaAc and HAC decreased bacterial diversity in biofilm.

As Decho (2000) documented cell within a biofilm can more easily adapt to changing environmental conditions. Figure 2.5 shows bacterial community compositions changed dramatically from day 0 to 2 at the all incubation condition. These were comparable with the study of Xue et al. (2012) that documented bacterial community composition dramatically changed within first 2 days.

2.4 Conclusions

This study implied that co-metabolic degradation of geosmin occurred in nature, because of the biofilm from the water treatment still had a capacity of geosmin

biodegradation in presence of NOM. Geosmin biodegradation was found to be enhanced as a co-metabolic biodegradation. Glucose and NaAc stimulated geosmin biodegradation, while HAC repressed geosmin biodegradation. Geosmin biodegradation seems to be induced by enzymes and cofactors produced by bacteria utilization of glucose and NaAc as energy source. Inhibition of HAC on geosmin biodegradation might be an evidence of hypothesis that geosmin biodegradation pathway is same as cyclohexanol.

Table 2.1 GC-MS condition for geosmin analysis

Gas Chromatography (GC) condition	
Column	Rix-5MS (RESTEK), 30 m x 0.25 mm ID x 0.25 μm fixed phase
Injection method	Splitless method high press mode 250 Kpa,1.5 min
Injection time	1 min
Sample volumn	1 μL
Vaporization Tem.	230°C
Oven condition	50°C ,1 min; 15°C /min; 250°C 3 min
Carries gas	He
Gas flow rate	Constant flow 5.19 cm/s
Mass spectrometry (MS) condition	
Ions face Tem.	230°C
Ions Tem.	200°C
Ionization method	SIM
Monitor ions	111, 112,125
Event time	0.2 s

Table 2.2 Primers and probe used in this study

	Sequences (5'-3')	References
Primers		
968F-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGG GCACGGGGGG-AACGCGAAGAACCTTAC	Valášková et al. (2009)
1401R	CGGTGTGTACAAGGCCCGGGAACG	
BACT1369F	CGGTGAATACGTTCYCGG	
PROK1492R	GGWTACCTTGTTACGACTT	Suzuki et al. (2000)
Probe		
TM1389BACT2	FAM-CTTGTACACACCGCCCGTC-TAMRA	

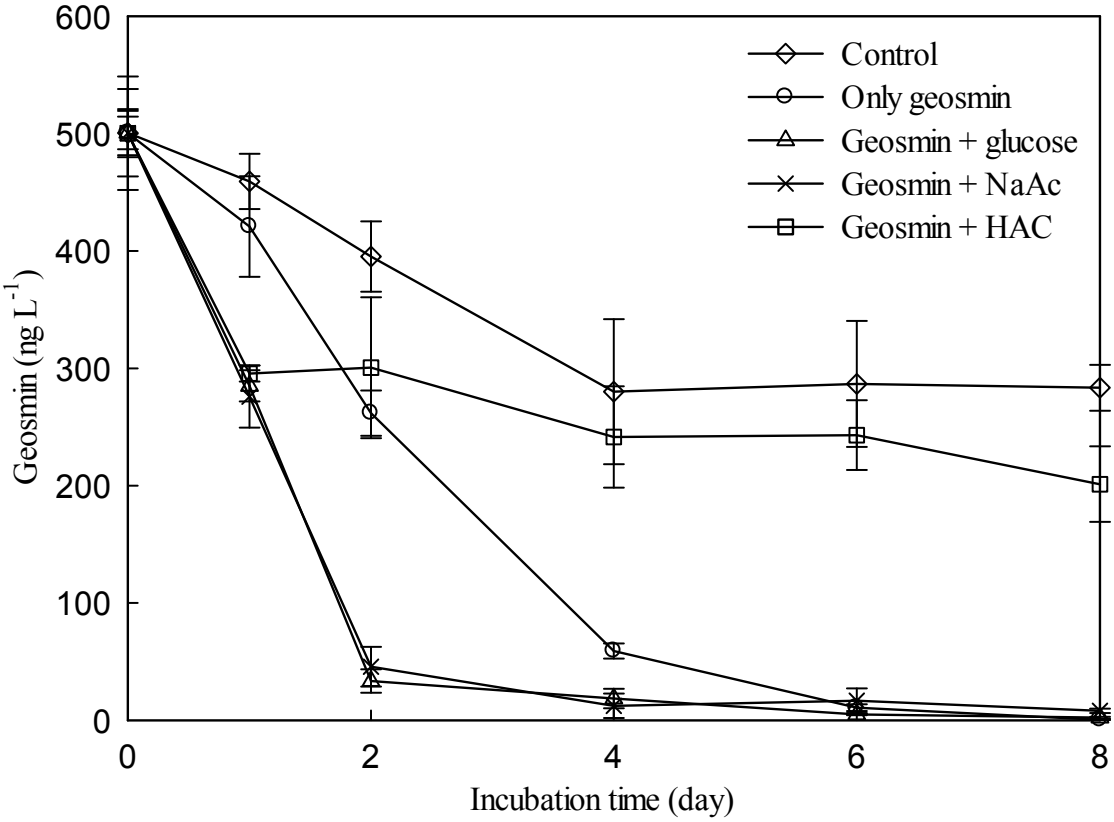


Fig. 2.1 Decrease in geosmin concentration during incubation of natural biofilm with several organic carbon source. ◊: Control; sterile biofilm with geosmin, ○: Only geosmin; biofilm with geosmin, Δ: Geosmin + glucose; biofilm with geosmin and glucose, ×: Geosmin + NaAc; biofilm with geosmin and NaAc, □: Geosmin + HAC; biofilm with geosmin and HAC. Bars represent the standard errors of the means for triplicates.

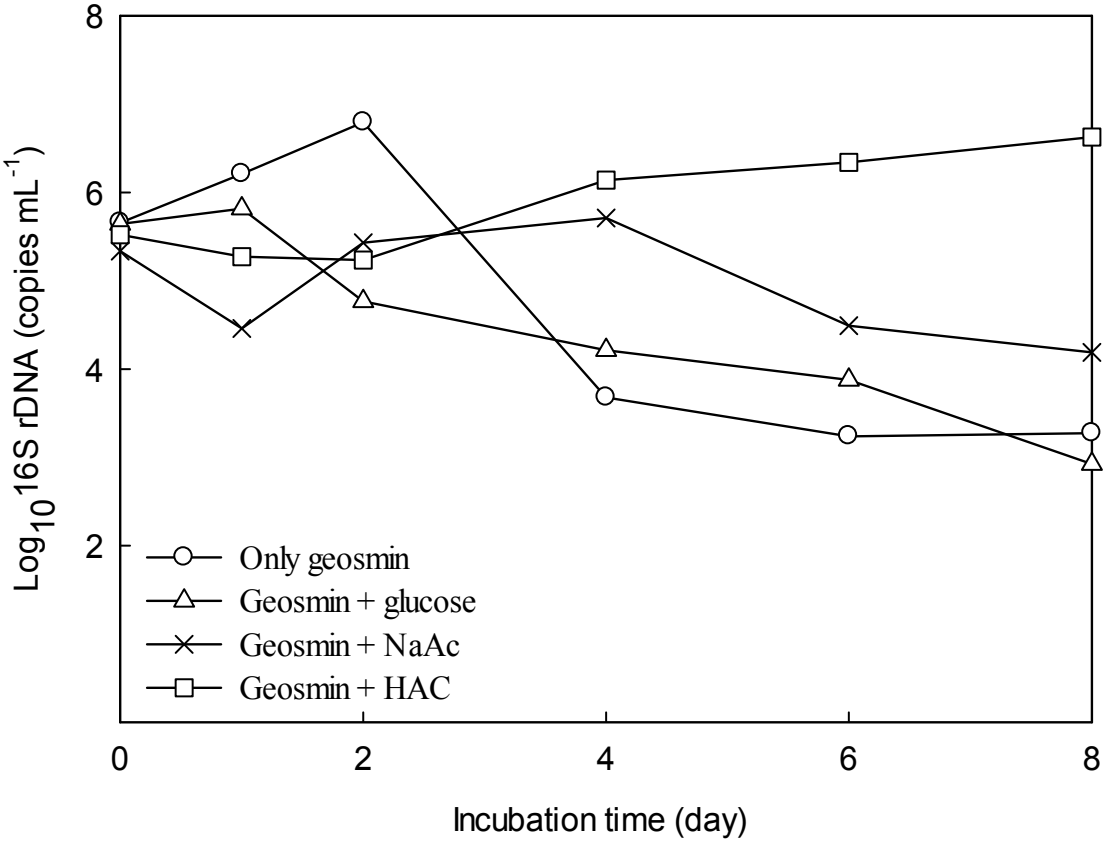


Fig. 2.2 Change of 16S rDNA copy number during incubation of natural biofilm with several organic carbon source. ○: Only geosmin; biofilm with geosmin, Δ: Geosmin + glucose; biofilm with geosmin and glucose, ×: Geosmin + NaAc; biofilm with geosmin and NaAc, □: Geosmin + HAC; biofilm with geosmin and HAC.

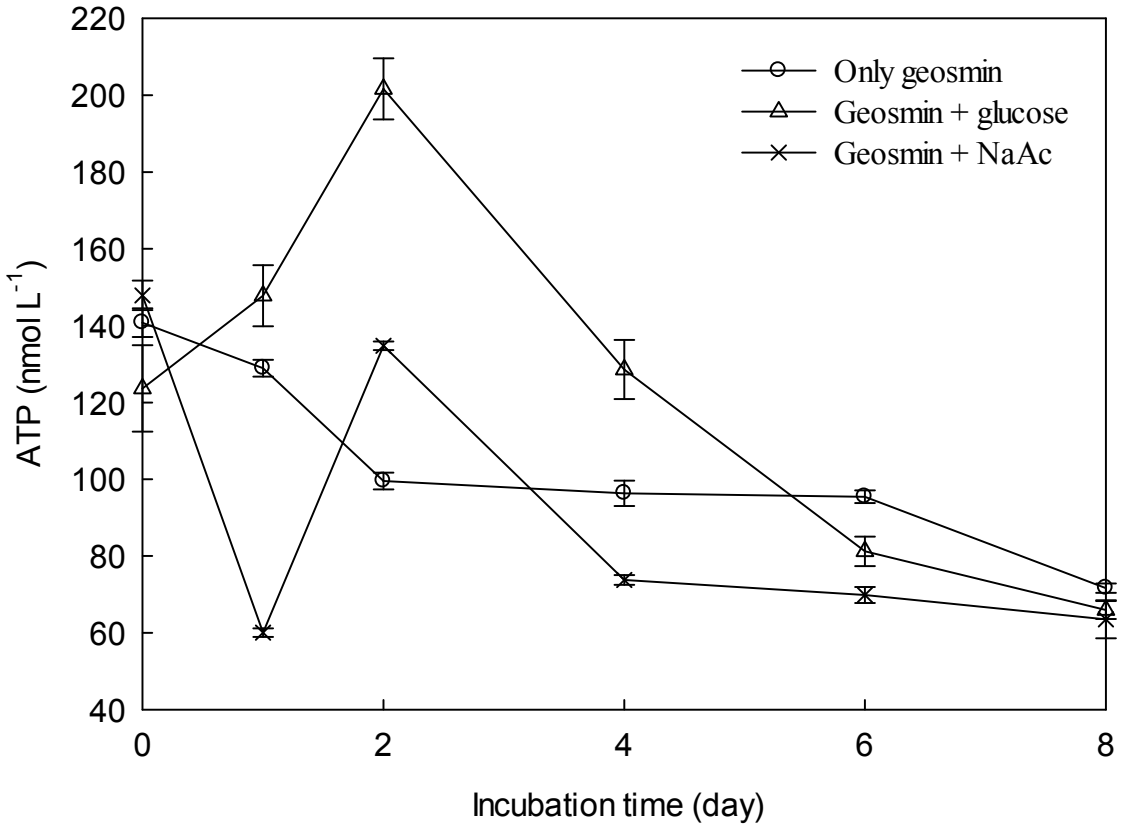


Fig. 2.3 Change of ATP concentration during incubation of natural biofilm with several organic carbon sources. ○: Only geosmin; biofilm with geosmin, Δ: Geosmin + glucose; biofilm with geosmin and glucose, ×: Geosmin + NaAc; biofilm with geosmin and NaAc. The ATP concentration of HAC was undetectable because of low pH from day 2. Bars represent the standard errors of the means for triplicates.

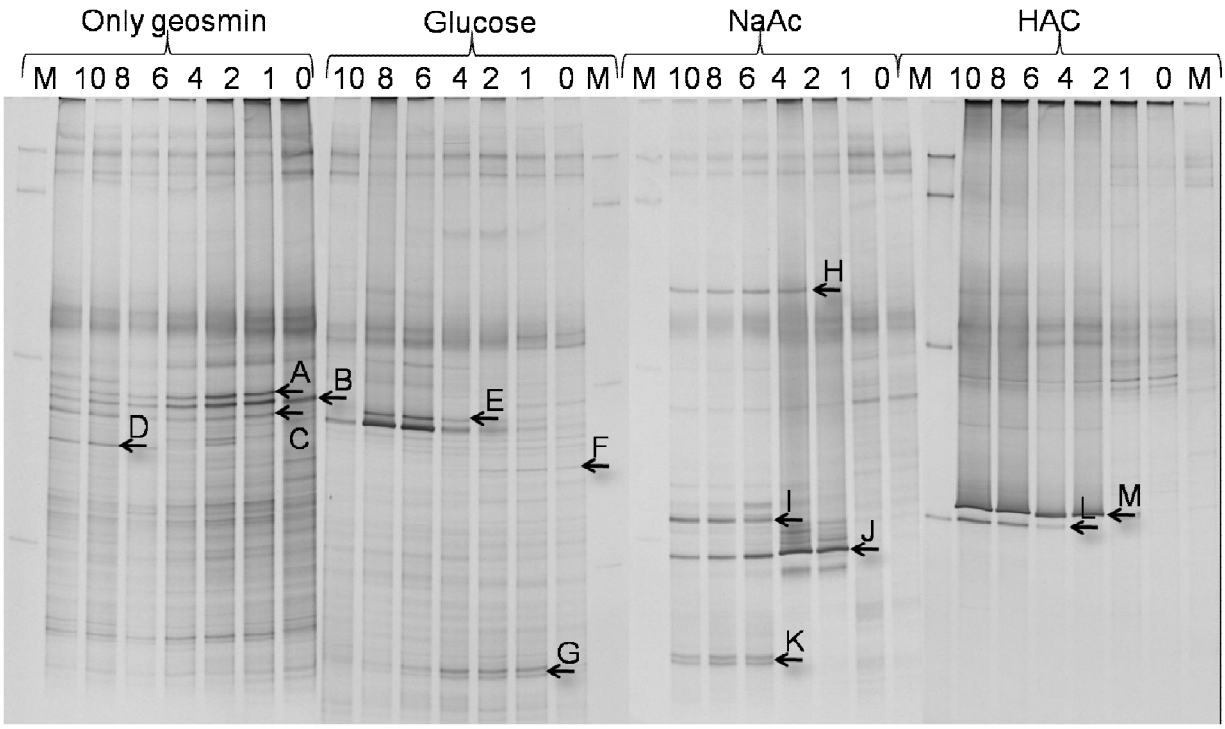


Fig. 2.4 DGGE band profiles of bacterial 16S rDNA fragments during geosmin degradation by microorganisms in the biofilm under only geosmin, glucose, NaAc and HAC conditions. 0-10 presents incubation day. M represents DGGE marker.

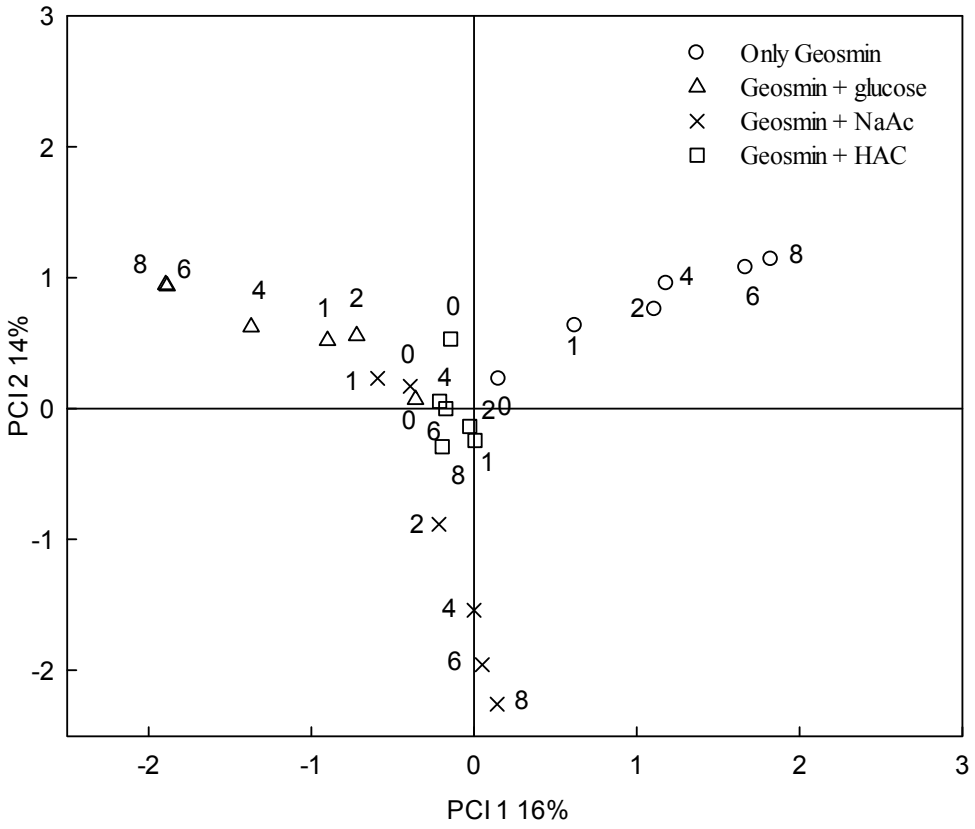


Fig. 2.5 Principal component analysis of the relative quantity generated by lane profiling for DGGE based upon 16S rDNA. PCI 1 represents the first principal component explaining 16% characteristic of samples. PCI 2 represents the second principal component explaining 14% characteristic of samples. PCI 1 and PCI 2 are uncorrelated. The number of 0, 1, 2, 4, 6, and 8 is incubation day. ○: Only geosmin; biofilm with geosmin, Δ: Geosmin + glucose; biofilm with geosmin and glucose, ×: Geosmin + NaAc; biofilm with geosmin and NaAc, □: Geosmin + HAC; biofilm with geosmin and HAC.

Chapter 3 Effect of inorganic nutrients (nitrate, ammonia and phosphate), dual nutrients substrance (glucose-NO_3^-, glucose-NH_4^+, HAC-NO_3^- and HAC-NH_4^+) on geosmin degradation by biofilm

3.1 Introduction

Cyanobacterial blooms occur particularly during a warmer weather and frequently trigger a high concentration of geosmin in water bodies (Medsker et al., 1968; Safferman et al., 1967). Consequently, it is increasing the enzymatic activity of geosmin degrading bacteria in submerged biofilm. Thus, much effort has been exerted to study about geosmin degradation capabilities of biofilm. However, the influence of external environmental conditions on the characteristics of microorganisms in the biofilm is scarce, potentially affecting geosmin degradation capacity of indigenous degraders. Moreover, it is noteworthy that considerable among of nutrients (especially organic carbon, nitrogen and phosphorous) supply to natural water. However, rare attention has been paid to compare the geosmin degradation in the presence or absence of organic, inorganic nutrients.

Elhadi et al. (2004) assessed that removal of geosmin by fresh and exhausted granular activated carbon (GAC) with adding cocktail nutrients solution. Experiments were conducted using two parallel filter columns containing fresh and exhausted GAC media and sand. As a result, fresh GAC showed total removals of geosmin ranged from 76% to 100%. The exhausted GAC initially removed less geosmin but it increased over time. This result showed nutrient and carbon source was important in

geosmin removal by biofilter. Researches about geosmin degradation indicated geosmin degradation was influenced by many factors, such as carbon source, necessary nutrient, temperature and initial geosmin concentration (Elhadi et al. 2006; Ho et al., 2007). For heterotrophic bacteria the empirical C: N ratio is very important for growtht. So the geosmin degradation potential of the microbial assemblages is susceptible to the fluctuation of environmental conditions. Particularly, it is noteworthy that available nutrients frequently supply to the water, together with geosmin (Li et al., 2011). Other researchers reported even small additions of organic substrates may trigger a shift in the composition of the microbial community and an accompanying change in the relative abundance of specific hydrolytic ectoenzymes (Eiler et al., 2003; Li et al., 2011). To date, the study about geosmin degradation performs with carbon source and nutrient substrates addition is scarce. Moreover little attention has been paid to combined action of carbon source and nutrient substrate to geosmin degradation by biofilm.

Efficient geosmin degradation depends upon not only natural organic matter, but also in-depth understanding on the variation in geosmin degradation with and without nutrients. To bridge the knowledge gaps, the main objective of this study was to investigate effect of inorganic nutrients (nitrogen, ammonia and phosphate), dual nutrients substance (glucose-NO_3^+, glucose-NH_4^-, HAC-NO_3^+ and HAC-NH_4^-) on geosmin degradation by biofilm. The sub-objective is to investigate winter biofilm geosmin degradation ability. The finally objective is to uncover which, carbon source or nutrient, was main factor effect geosmin degradation.

3.2 Materials and methods

3.2.1 Chemical

Geosmin standard material was purchased from WAKO pure Chemicals Ltd. Osaka, Japan. The authentic sample of 20 mg was dissolved in ultra pure water (Resistivity 18.0 MΩ·cm at 25°C) prepared with a water purification system (Purelite PRB-001A/002A) supplied by Organo, Japan. The stock solution was transferred into brown air-tight glass bottle and stored in the dark at 4°C prior to use.

3.2.2 Batch biodegradation experiments

Natural biofilm formed in the honeycomb tube was sampled from a water treatment plant at Lake Kasumigaura in December 2012. The biofilm was scraped from the surface of honeycomb by sterile ladle. The sampled biofilm in sterile centrifuge tube (15 mL) was stored in 4°C ice box before use.

The effect of nutrient on geosmin biodegradation tests were conducted in 1000 mL conical flasks. In each flask, biofilm (fresh, 1.0 g wet) was added to 500 mL sterile ultra pure water. After adding biofilm into flask, base water contents were analyzed and total phosphorus, COD_{Mn} and BOD_5 were 0.077, 1.10 and 0.43 mg L^{-1}, respectively, and total nitrogen was as lower as 0.02 mg L^{-1}. Cultures were spiked with geosmin to establish an initial concentration of 500 ng L^{-1}, which is the average geosmin concentration occurring at Lake Kasumigaura in water. The effect of nutrient substrates on geosmin biodegradation was investigated by addition of ammonium

chloride (NH_4^+), sodium nitrate (NO_3^-) and potassium dihydrogen phosphate (PO_4^{3-}). The initial concentration of ammonium chloride, sodium nitrate and potassium dihydrogen phosphate were, 1 mg L^{-1}, 1mg L^{-1} and 1 mg L^{-1}, respectively. The flasks were stopped with silicone stopper, and incubated in a constant rotation incubator (25°C, 100 rpm). A subsample of 15 mL was removed from each flask at 0, 1st, 2nd, 4th, 6th, 8th 10th day for determination geosmin, pH, bacterial activity, and bacterial abundance and bacterial community structures.

The co-action effect of carbon and inorganic nutrient (nitrogen) on geosmin biodegradation was done as following (detail list in Table 3.1). Fresh biofilm (6 g wet) was aseptically added into 60 mL of sterile ultra pure water. Then the biofilm was thoroughly homogenized, and divided into 4 conical flasks (each 15 mL into 1000 mL flask). Geosmin was added to every flask to form initial concentration of 500 ng L^{-1} which is the average geosmin concentration occurring at Lake Kasumigaura in water. Ammonium chloride (NH_4^+), and glucose or acetic acid (HAC) were added to 2 flasks, its initial concentrations were 18.7 mol N (NH_4^+) L^{-1}, 33.3 mol C (glucose) L^{-1} or 24.3 mol C (HAC) L^{-1} respectively. Sodium nitrate (NO_3^-) and glucose or acetic acid (HAC) were added to 2 flasks, its initial concentrations were 11.8 mol N (NO_3^-) L^{-1}, 33.3 mol C (glucose) L^{-1} or 24.3 mol C (HAC) L^{-1}, respectively. Finally, the flasks were stopped with silicone stopper and incubated in a constant rotation incubator (25°C, 100 rpm). A subsample of 15 mL was removed from each flask at 0, 1st, 2nd, 4th, 6th, 8th and 10th day for determination geosmim concentration, ATP concentration, and bacterial number.

The control was conducted which contained sterile biofilm and geosmin to investigate abiotic impact. Also only geosmin condition which consisted of bioflim and geosmin investigated geosmin degradation as a criterion.

3.2.3 Geosmin analysis

Hexane extraction analysis for geosmin and MIB (Jensen et al., 1994) was used to extract the geosmin. Progress of geosmin biodegradation in each flask was monitored by GC/MS analysis in a 2010 plus GC/MS (SHIMADZU, Japan) equipped with a RESTEK Rix–5MS capillary column (30 m x 0.25 mm ID x 0.25 μm fixed phase) with helium as carrier gas. GC-MS condition was list in Table 2.1.

3.2.4 DAPI direct counting

The number of bacterial was determined from subsamples fixed with 10% formalin solution (1% final concentration) and stained with 4', 6-diamidino-2-phenylindole (DAPI; 0.5 μg mL^{-1} final concentration) for 10 min after filtration onto 0.2 μm white polycarbonate filter (Whatmann). The filters were rinsed with 0.2 μm filtered distilled water and mounted on glass slides with non-fluorescent immersion oil before counting. The slides were kept frozen at –30°C until microscopic analyses were done. The filters were inspected under an Olympus BX-50 microscope, equipped with an ultraviolet and blue filter set with excitation and emission wavelengths of 365 and 390 nm, at 1000× magnification (Christoffersen et al., 2002).

3.2.5 ATP analysis

The ATP standard curve was prepared from a serial dilution of standard ATP solution, results of linear between 1 μM to 10 pM. Mixing 100 μL BacTiter-Glo^{TM} Reagent and 100 μL water samples or standard in each hole of an opaque-walled multiwall plate and incubated for 1 min to insure no lysis required to release ATP. Then luminescence was recorded by Fluoroskan Ascent type 374 multi-well plate reader (Labsystems, Helsinki, Finland).

3.2.6 PCR-DGGE analysis

Total DNA extraction was performed according to phenol–chloroform extraction method (Chomczynski and Sacchi,1987), and PCR-DGGE analysis was done as the same method in Chapter 2 (2.2.6).

3.2.7 Statistics analysis

Principal component in the statistical Package for Social Science v17.0 software (SPSS Inc IL, USA) was applied to analyze principal components of DGGE fingerprint. Statistical significance was accepted at $P<0.05$. Bacterial diversity index (*IB*) was calculated as equation (2-1).

3.3 Results and discussion

Figure 3.1 showed change of geosmin concentrations during incubation under NO_3^-, NH_4^+, and PO_4^{3-} adding conditions. There was some loss of geosmin (41.7%) in

control that implied geosmin was removed through the abiotic factors. Geosmin degradation started immediately from the beginning of experiment. Geosmin degraded during initial 4 days at only geosmin condition, but there were no increase of bacterial number (Fig. 3.2) and activity (Fig. 3.3). Total of 80.6% geosmin was degraded by day 10, which indicated that geosmin degradation bacteria were in the biofilm in winter. Compared to the result of Chapter 2 experiment (using autumn biofilm), geosmin degradation efficiency was lower. The reason of this low efficiency was reported by Xue et al. (2012). They assessed geosmin removal by natural biofilm during a year; the results indicated that geosmin degradation rate changed with season. Spring biofilm indicated significant geosmin removal efficiency. Winter biofilm indicated the lowest geosmin removal efficiency. Obviously, autumn biofilm had been exposed to geosmin in natural water during spring and summer of 2012. Hence, exposure to geosmin may stimulate enzymatic activity of geosmin degradation in autumn biofilm. These observations would be implied that the geosmin degradation capability of biofilm. Also geosmin degradation by biofilm might be influenced by history of geosmin exposure.

Geosmin degradation rate constant of only geosmin was 0.16 d^{-1} (R^2=0.94), and it was a pseudo-first-order reaction. To date, geosmin degradation mechanism reported were all co-metabolism no matter by biofilm or pure strain; this might be the reason why mechanism of geosmin degradation by bacteria was still unknown and why there were various geosmin reaction kinetics. So geosmin degrading enzyme might be produced when primary substrate was utilized by bacterial and this enzyme might be

common.

Bacterial cell density of only geosmin condition increased from 0 to 6th day, then decreased gradually during incubation period (Fig. 3.2 and 3.6). Change of ATP concentration had a clear correlation with geosmin concentration under only geosmin condition (Fig 3.3 and Fig. 3.7). For only geosmin, band D (Fig. 3.4) and band B (Fig. 3.9 and 3.11) disappeared from the 6th day. Band C appeared from the 6th day until end of experiment which might be the geosmin-degrading bacterium. These implied that bacterial community composition of only geosmin evidently changed from the 6th day. The (*IB*) value of only geosmin was 0.17 at initial day and gradually decreased to 0.04, which implied that geosmin induced bacterial diversity decreased that was due to bacteria possessed capacity of geosmin degrading in the biofilm.

3.3.1 Effect of inorganic nitrogen on geosmin degradation

As Figure 3.1 shown, geosmin degradation was stimulated by NO_3^-. The fastest geosmin degradation occurred in first 4 days which resulted from increase of ATP concentration and distinct increase of bacterial number. Bacterial number (Fig. 3.2) and ATP concentration (Fig. 3.3) reached to as high as 1.47×10^7 cell and 108.65 nmol mL^{-1} within first 4 days, respectively. Geosmin was completely degraded and geosmin removal efficiency was 99% (including abiotic impact) by the tenth day. This was likely to imply NO_3^- stimulated the bacterial number and activity. Rate constant was 0.37 d^{-1} (R^2= 0.97) under NO_3^- condition which was 2 times higher than only geosmin. This high rate constant was attributed to stimulation of ATP production and bacterial

growth. However, this rate constant was lower than only geosmin in Chapter 2 result, which indicated that necessary nutrient for bacterial growth in MSM, except for nitrogen, also influenced geosmin biodegradation.

NH_4^+ also stimulated geosmin degradation (Fig. 3.1). The removal efficient was 99% the same as NO_3^-, but rate constant and complete biodegradation time was different. Rate constant of NH_4^+ (0.59 d^{-1} (R^2= 0.93)) was higher than NO_3^-, and geosmin was completely degraded by the 8th day. Corresponding to rapid geosmin degradation, bacterial activity reached to 124.9 nmol mL^{-1} on the 4th day. This result implied that rapid geosmin degradation was caused by high bacterial activity. Bacteria utilized ammonia to synthesis amino acids, which are the building blocks of protein.

Figure 3.4 showed that the DGGE fingerprint of only geosmin NO_3^-, NH_4^+ and PO_4^{3-} on the 0th, 1st, 2nd, 4th, 6th, 8th and 10th day. There were no obvious dominant species in NO_3^-. The *IB* value of NO_3^- kept stable during geosmin degradation at about 0.16. Under NH_4^+ condition, the two dominant bacteria appeared from the 2nd day. These implied that community composition significantly changed from the 2nd day and kept stable by the end of incubation time. The bacterial diversity of NH_4^+ decreased from the 2nd day which illustrated that NH_4^+ influenced bacterial diversity.

Figure 3.5 showed that principal component analysis of the relative quantity generated by lane profiling of DGGE based upon 16S rDNA. Each day's point of only geosmin were located in closeness area, which implied geosmin affected community composition but not so significant. Samples of

Bacterial community composition of NH_4^+ evidently changed between the 2nd and

4th day. At the 1st day, the plot position of NH_4^+ condition was located in upper area while the 1st day's plot position of NO_3^- was located in left part. These differences indicated that NO_3^- and NH_4^+ had obvious impact on bacterial community compositions and effects NO_3^- and NH_4^+ for geosmin biodegradation by biofilm were different from each other.

Several investigators had demonstrated that bacterial growth in aquatic ecosystems could be limited by the availability of nitrogen and phosphorous (Carlsson and Caron, 2001). Stimulation of NO_3^- and NH_4^+ on geosmin degradation implied that, as common nutrient substrates in aquatic environment, nitrate and ammonia could be important factors affecting geosmin biodegradation. As Li et al. (2011) reported that microcystin-LR degradation was inhibited by adding ammonia at concentration of 100 mg L^{-1} and 1000 mg L^{-1}, while it was stimulated by adding nitrate at concentration of 100 mg L^{-1} and 1000 mg L^{-1}. These two results showed that nitrate and ammonia could stimulate biodegradation, however the contributing rate depended on each concentration.

3.3.2 Effect of potassium dihydrogen phosphate on geosmin degradation

Geosmin degradation was enhanced by potassium dihydrogen phosphate during degradation period (Fig. 3.1). Geosmin degradation was rapid in the first day and kept stable from the 2th day. The rate constant of geosmin degradation of PO_4^{3-} was 0.93 d^{-1} (R^2=0.99). Although this rate constant was higher than other conditions, this rate constant just contained three data points based on the concept of pseudo-first-order

reaction.

Microorganisms required phosphorus in the biosynthesis of nucleic acids (DNA, RNA), ATP and other cellular components. The bacterial number gradually increased until end of experiment, but this increase was very slight. While bacterial activity was stimulated by phosphate, which implied that bacteria used phosphate to synthesis ATP.

Geosmin degradation mechanism was co-metabolism which was reported indirectly by other researchers (Ho et al., 2007; Saito et al., 1999). As co-metabolism defined that transformation of a non-growth substrate by growing cells in the presence of a growth substrate, geosmin was the non-growth substrate because no increase of bacterial number and activity were observed during geosmin degradation in only geosmin, it was implied that geosmin could not be utilized by bacteria to proliferate and produce energy. The phosphorus was utilized by bacteria to growth or keep life. Bacterial activity increased within first 1 day, decreased on the 2^{nd} day (Fig.3.3). This was corresponding to rapid geosmin degradation in first day, and then decreased from the 2^{nd} day. These results seemed that phosphorus stimulated geosmin degradation by increasing bacterial activity.

For PO_4^{3-}, there were many bands in initial day (Fig.3.4). Bands I and K appeared on the 6^{th} day. Bands H and J appeared on the 8^{th} day. These implied that bacterial community composition obviously changed between the 6^{th} and 8^{th} day. Bacterial diversity index *IB* increased from 0.12 at initial day to 0.25 on 10^{th} day. Samples of PO_4^{3-} also were located in closeness area and almost the same as only geosmin. It could be concluded that PO_4^{3-} had no significant effect on bacterial community

composition, but the bacterial number and activity increased during rapid geosmin degradation period. This result illustrated that PO_4^{3-} stimulated geosmin degradation capacity of bacteria.

Comparing with geosmin degradation capacity of autumn biofilm, winter biofilm possessed low geosmin degradation capacity. The effect of nutrient on geosmin biodegradation was not as significant as carbon source. However because medium used in this two chapter study was different, it could not definitely concluded that effect of carbon source on geosmin biodegradation was significant than inorganic nutrients.

3.3.3 Effect of glucose-NO_3^- and glucose-NH_4^+ on geosmin degradation

Geosmin degradation was enhanced under glucose-NO_3^- and glucose-NH_4^+ condition (Fig. 3.6), which indicated that geosmin degradation was stimulated by glucose-NO_3^- and glucose-NH_4^+. Geosmin was completely degraded by the 10^{th} day; nevertheless geosmin was not entirely removed by biofilm at 10^{th} day in only geosmin condition. This difference might be caused by different of bacterial cell densities and activities. Under glucose-NH_4^+ condition, bacterial cell density gradually increased and reached as high as 1.60×10^7 cell mL^{-1} on the 6^{th} day. Bacterial activity reached the peak of 212.35 nmol mL^{-1} on the 4^{th} day. Under glucose-NO_3^- condition, bacterial activity reached peak also on the 4^{th} day, but a little lower than glcose-NH_4^+. Geosmin degradation rate constant was 0.34 d^{-1} (R^2=0.93) for glucose-NO_3^-, 0.39 d^{-1} (R^2=0.96) for glucose-NH_4^+, respectively.

Under glucose-NO_3^- condition, dominant species decreased from the 1st day to the 4th day and were stable until end of experiment (Fig.3.9). It was indicated that glucose-NO_3^- had significant impact on changing bacterial community compositions during day 1 and 4. Then, the composition of glucose-NO_3^- was stable by the end of incubation period. The *IB* value had a slight fluctuation and was about 0.14. On the other hand, bacterial community composition in glucose-NH_4^+ decreased with time (Fig.3.9). The *IB* value decreased from the 1st day and reached to 0.45 by the end of experiment, this value was higher than that of glucose-NO_3^- value. This revealed that NO_3^- and NH_4^+ had different effect on bacterial community compositions.

Figure 3.10 showed that principal component analysis of the relative quantity of band generated by lane profile for DGGE based upon 16S rDNA. Samples of glucose-NO_3^- were located in almost same area except for day 0 and 1. Bacterial community composition of glucose-NO_3^- obviously changed during the first 2 days, and then was located in same area. While glucose-NH_4^+ bacterial community composition changed with time and was significantly different from only geosmin and glucose-NO_3^-. These results showed that NO_3^- and NH_4^+ had different impact on bacterial community composition.

3.3.4 Effect of HAC-NO_3^- and HAC-NH_4^+ on geosmin degradation

Interestingly, the effect of HAC-NO_3^- and HAC-NH_4^+ on geosmin degradation differed from glucose-NO_3^- and glucose-NH_4^+. Under HAC-NH_4^+ and HAC-NO_3^- condition, 79% and 59% of initial geosmin had been degraded by the 10th day,

respectively (Fig.3.6). This suggested that the geosmin degradation was repressed by HAC-NO_3^-, although this effect was not statistically significant (p>0.05). However, geosmin removal ratio was same as only geosmin under HAC-NH_4^+ condition. The geosmin degradation rate constant was 0.21 d^{-1} (R^2=0.91) for HAC-NO_3^- and 0.15 d^{-1} (R^2=0.92) for HAC-NH_4^+, respectively. Bacterial cell densities of HAC-NO_3^- and HAC-NH_4^+ were no fluctuation during the experiment (Fig.3.7). ATP concentration could not be detectable because of limitation of measurement method.

Figure 3.11 shows that dominant bacteria did not appear when HAC-NO_3^- and HAC-NH_4^+ were added. No obvious band appeared and disappeared. But compared to HAC-NH_4^+, HAC-NO_3^- had many bands. The *IB* value of HAC-NH_4^+ was lower than 0.1, while the *IB* value of HAC-NO_3^- was higher than 0.3, which implied that NH_4^+ and NO_3^- had different impact on bacterial community compositions.

Figure 3.12 shows that principal component analysis of the relative quantity generated by lane profiling for DGGE based upon 16S rDNA. In the first 2 days community composition of HAC-NH_4^+ obviously changed and was stable from the 4^{th} day. All plots were located in left area of the figure, while the plots of HAC-NO_3^- were located in upper area of the figure (Fig. 3.12). Community composition of HAC-NO_3^- changed with time and the compositions of HAC-NO_3^- and HAC-NH_4^+ was different from each other.

In this chapter, carbon source was the main factor influenced geosmin degradation. This result was in accordance with result of chapter 2. Chapter 2 reported that glucose stimulated geosmin degradation, while HAC inhibited geosmin degradation. This

might be bacteria using carbon source to synthesize ATP and then enhanced geosmin degradation, while nutrient is constituent of cell, like amino acids and nucleic acid. Bacteria could not produce energy from nutrient, but some bacteria could use nitrogen to synthesize coenzymes, which also could stimulate geosmin degradation.

3.4 Conclusions

The winter biofilm could effectively degrade geosmin under only geosmin and inorganic nutrients added conditions. The geosmin degradation was accelerated by nitrate, ammonia, phosphate, glucose-NO_3^- and glucose-NH_4^+. However, the influence of HAC-NO_3^- and HAC-NH_4^+ on geosmin degradation was not significant. The phosphate had most significant effect on stimulating geosmin degradation. Bacteria could utilize carbon and nutrient to synthesize some cofactors or enzymes to enhance geosmin degradation.

Table 3.1 Culture components used in this study

Items	Glucose-NH_4^+	Glucose-NO_3^-	HAC-NH_4^+	HAC-NO_3^-	Only geosmin	Control
Glucose (33.3 mol L^{-1})	○	○			○	
Acetic acid (24.3 mol L^{-1})			○	○		
Ammonia chloride (18.7 mol L^{-1})	○		○			
Sodium nitrate (11.8 mol L^{-1})		○		○		
Geosmin (500 ng L^{-1})	○	○	○	○	○	○
Bioflim (1 g)	Fresh	Fresh	Fresh	Fresh	Fresh	Inactive

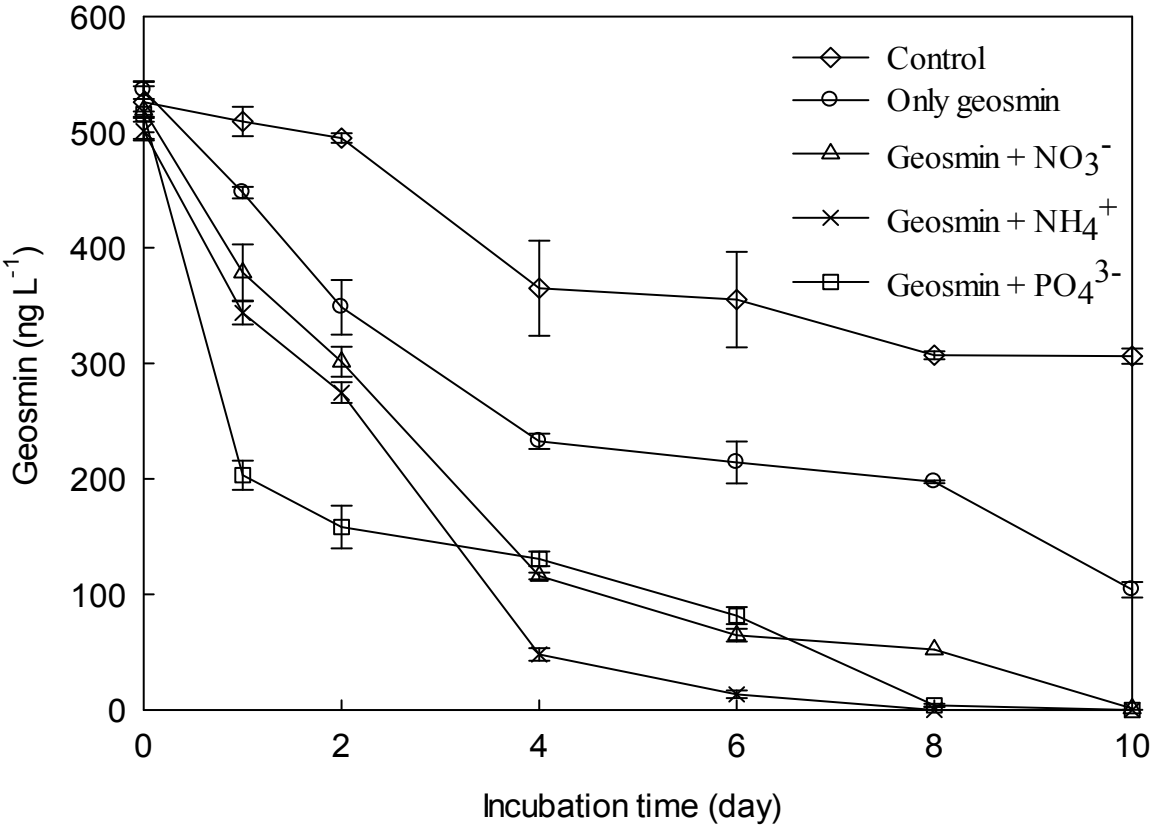


Fig. 3.1 Decrease in geosmin concentration during incubation of natural biofilm with several inorganic nutrients sources. ◊: Control; sterile biofilm with geosmin, ○: Only geosmin; biofilm with geosmin, Δ: Geosmin + NO_3^-; biofilm with geosmin and nitrate, ×: Geosmin + NH_4^+; biofilm with geosmin and ammonia, □: Geosmin + PO_4^{3-}; biofilm with geosmin and phosphate. Bars represent the standard errors of the means for triplicates.

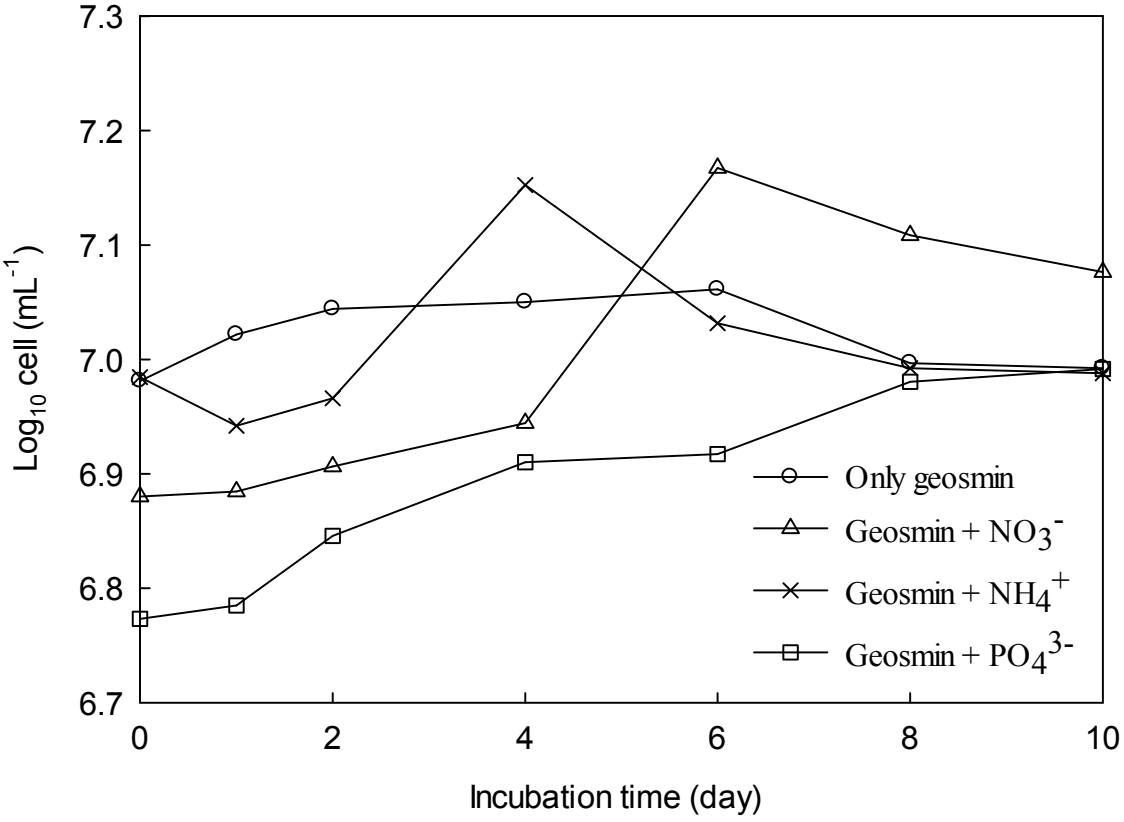


Fig. 3.2 Change of bacterial cell density during incubation of natural biofilm with several inorganic nutrients sources. ○: Only geosmin; biofilm with geosmin, Δ: Geosmin + NO_3^-; biofilm with geosmin and nitrate, ×: Geosmin + NH_4^+; biofilm with geosmin and ammonia, □: Geosmin + PO_4^{3-}; biofilm with geosmin and phosphate.

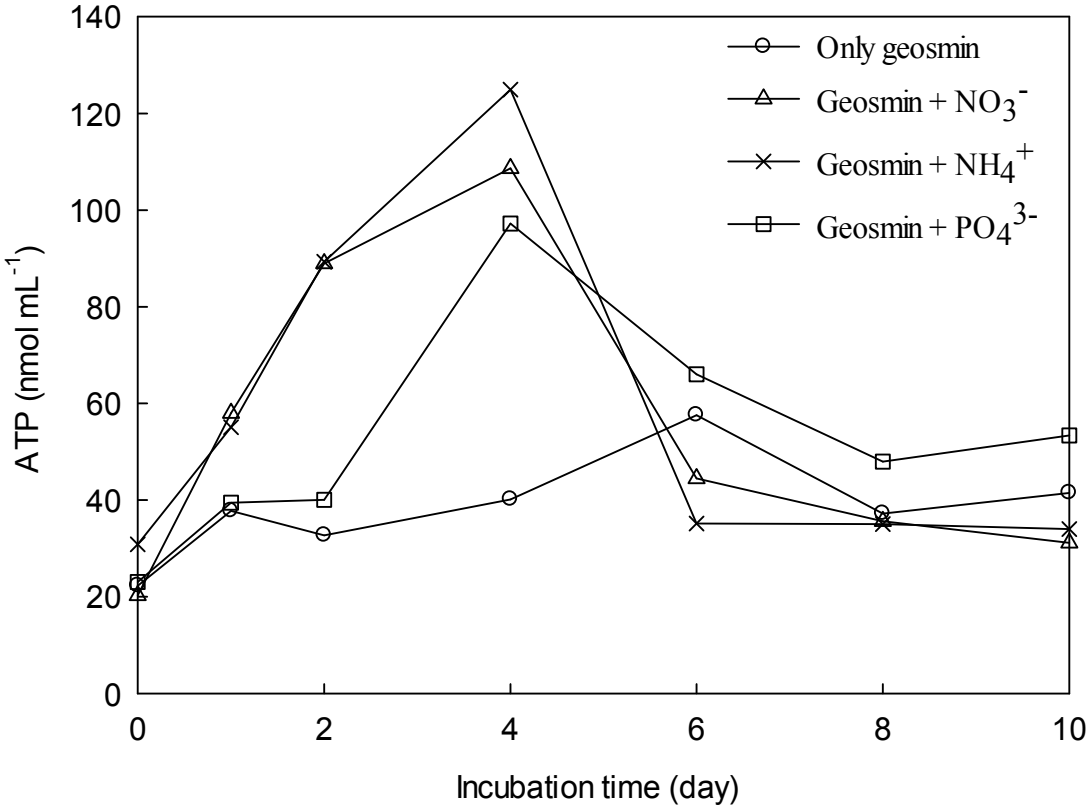


Fig. 3.3 Change of ATP concentration during incubation of natural biofilm with several inorganic nutrients sources. ○: Only geosmin; biofilm with geosmin, Δ: Geosmin + NO_3^-; biofilm with geosmin and nitrate, ×: Geosmin + NH_4^+; biofilm with geosmin and ammonia, □: Geosmin + PO_4^{3-}; biofilm with geosmin and phosphate.

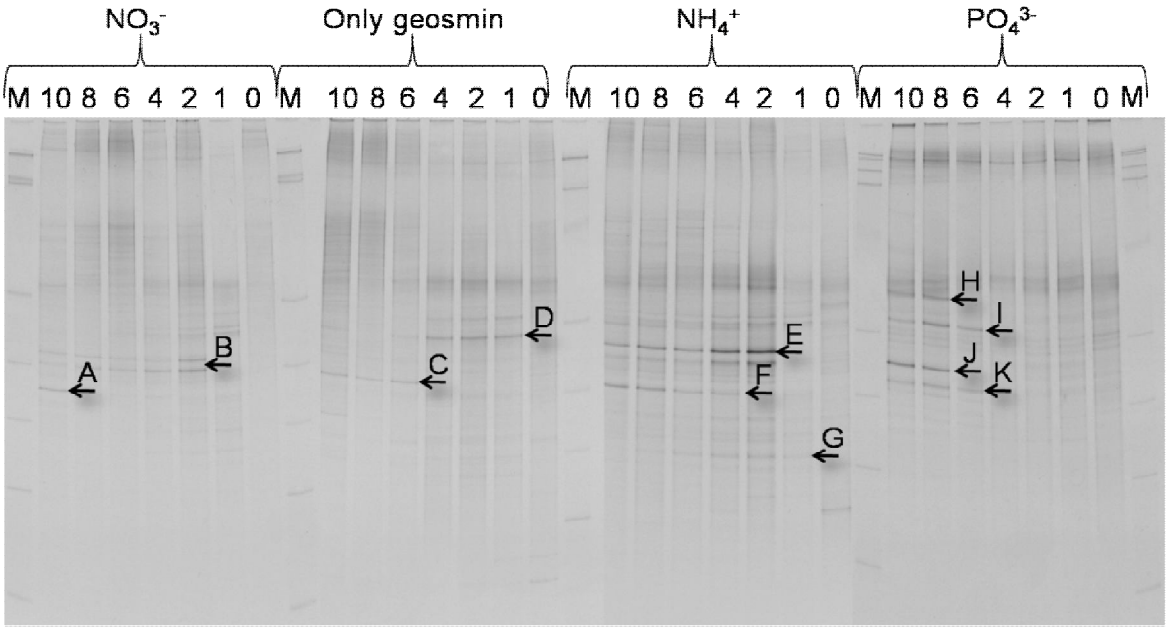


Fig. 3.4 DGGE band profiles of bacterial 16S rDNA fragments during geosmin degradation by microorganisms in the biofilm under only geosmin, NO_3^-, NH_4^+ and PO_4^{3-} conditions. 0-10 presents samples taking day. M represents DGGE marker.

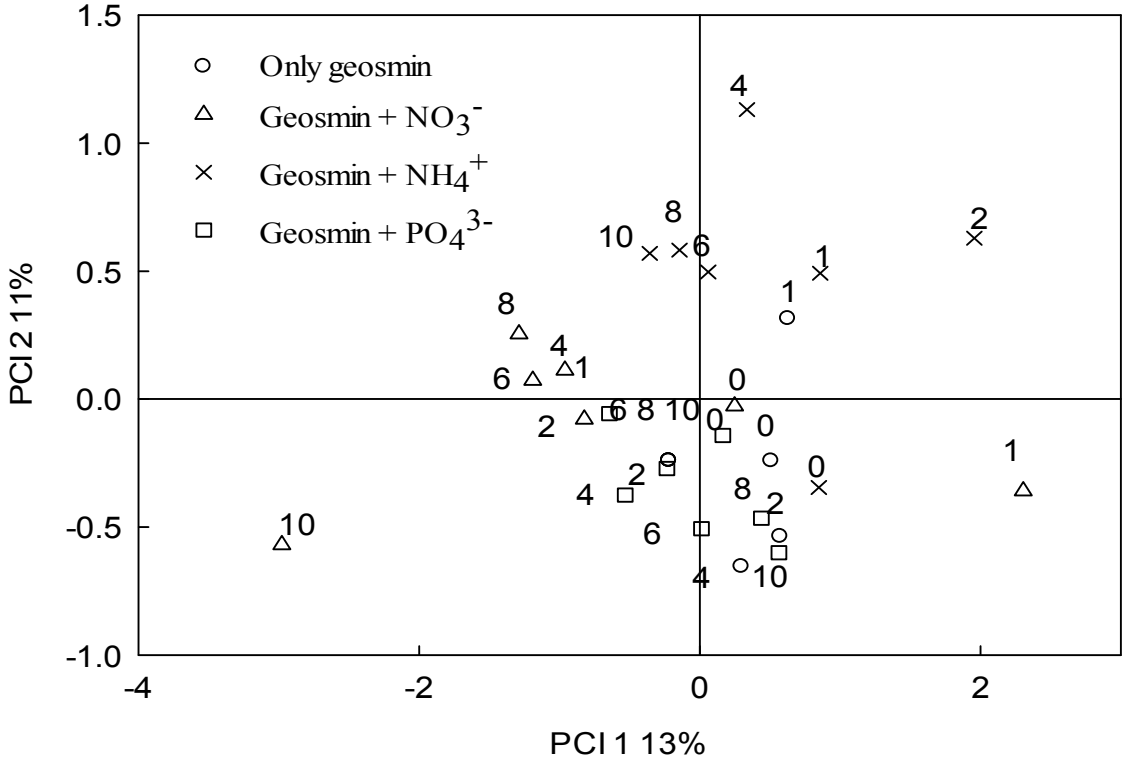


Fig. 3.5 Principal component analysis of the relative quantity generated by lane profiling for DGGE based upon 16S rDNA. PCI 1 represents the first principal component explaining 13% characteristic of samples. PCI 2 represents the second principal component explaining 11% characteristic of samples. PCI 1 and PCI 2 are uncorrelated. The number of 0, 1, 2, 4, 6, 8 and 10 is incubation day. ○: Only geosmin; biofilm with geosmin, Δ: Geosmin + NO_3^-; biofilm with geosmin and nitrate, ×: Geosmin + NH_4^+; biofilm with geosmin and ammonia, □: Geosmin + PO_4^{3-}; biofilm with geosmin and phosphate.

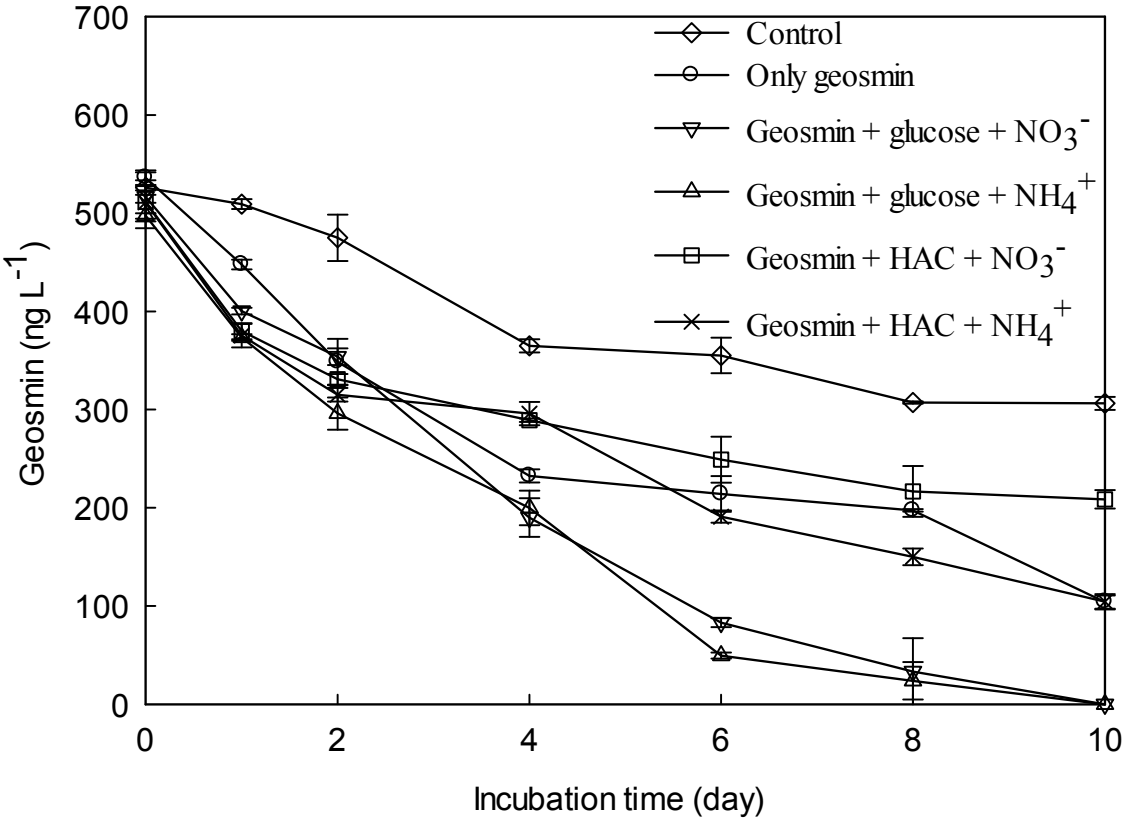


Fig. 3.6 Decrease in geosmin concentration during incubation of natural biofilm with several dual nutrients sources. ◊: Control; sterile biofilm and geosmin, ○: Only geosmin; biofilm and geosmin, ▽: Geosmin + glucose + NO_3^-; biofilm with geosmin, glucose and nitrate, Δ: Geosmin + glucose + NH_4^+; biofilm with geosmin, glucose and ammonia, □: Geosmin + HAC + NO_3^-; biofilm with geosmin, HAC and ammonia, ×: Geosmin + HAC + NH_4^+; biofilm with geosmin, HAC and ammonia, Bars represent the standard errors of the means for triplicates.

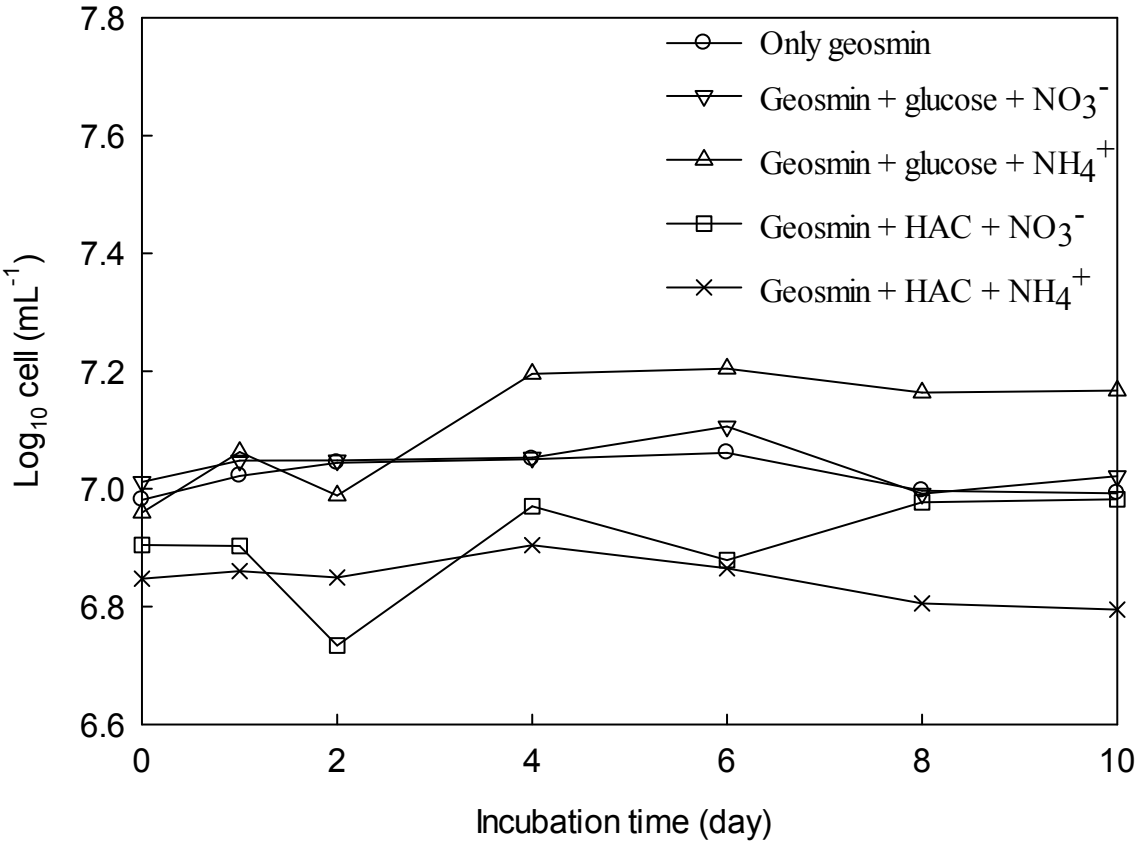


Fig. 3.7 Change of bacterial cell density during incubation of natural biofilm with several dual nutrients sources. ○: Only geosmin; biofilm and geosmin, ▽: Geosmin + glucose + NO_3^-; biofilm with geosmin, glucose and nitrate, Δ: Geosmin + glucose + NH_4^+; biofilm with geosmin, glucose and ammonia, □: Geosmin + HAC + NO_3^-; biofilm with geosmin, HAC and ammonia, ×: Geosmin + HAC + NH_4^+; biofilm with geosmin, HAC and ammonia.

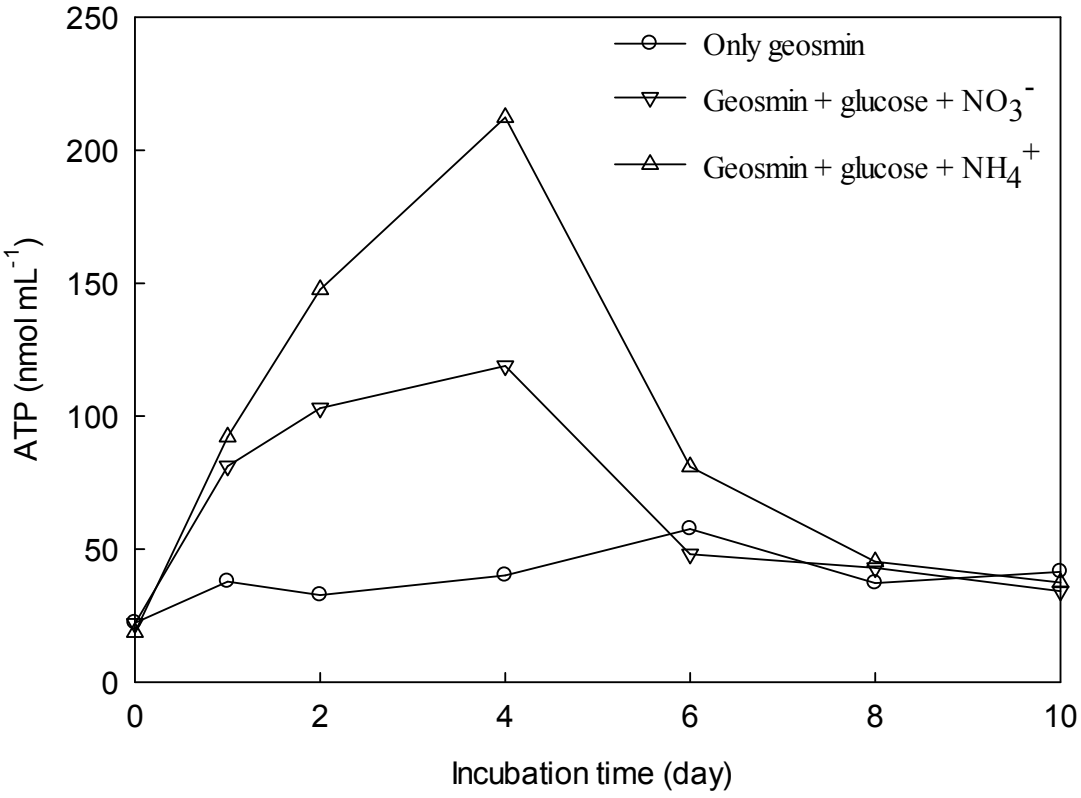
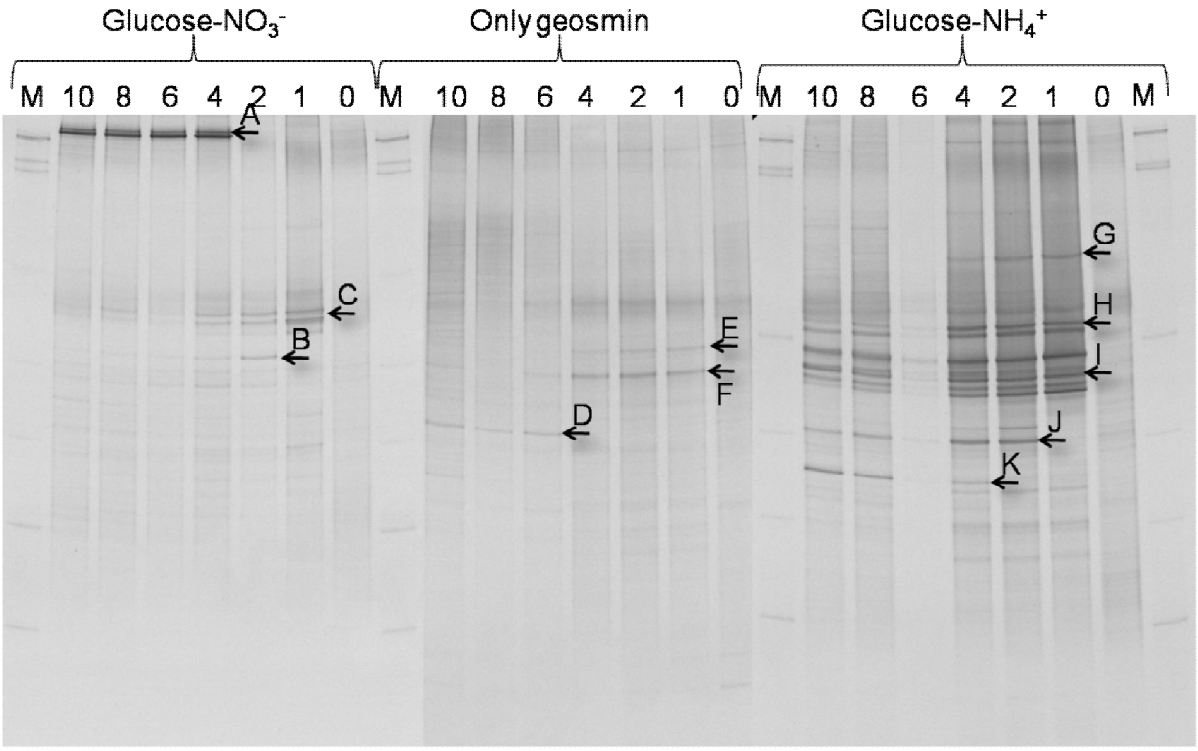


Fig. 3.8 Change of ATP concentration during incubation of natural biofilm with several dual nutrients sources. ○: Only geosmin; biofilm and geosmin, ▽: Geosmin + glucose + NO_3^-; biofilm with geosmin, glucose and nitrate, Δ: Geosmin + glucose + NH_4^+; biofilm with geosmin, glucose and ammonia.

Fig. 3.9 DGGE band profiles of bacterial 16S rDNA fragments during geosmin



degradation by microorganisms in the biofilm under only geosmin, glucose-NH_4^+ and glucose-NO_3^- conditions. 0-10 presents samples taking day. M represents DGGE marker.

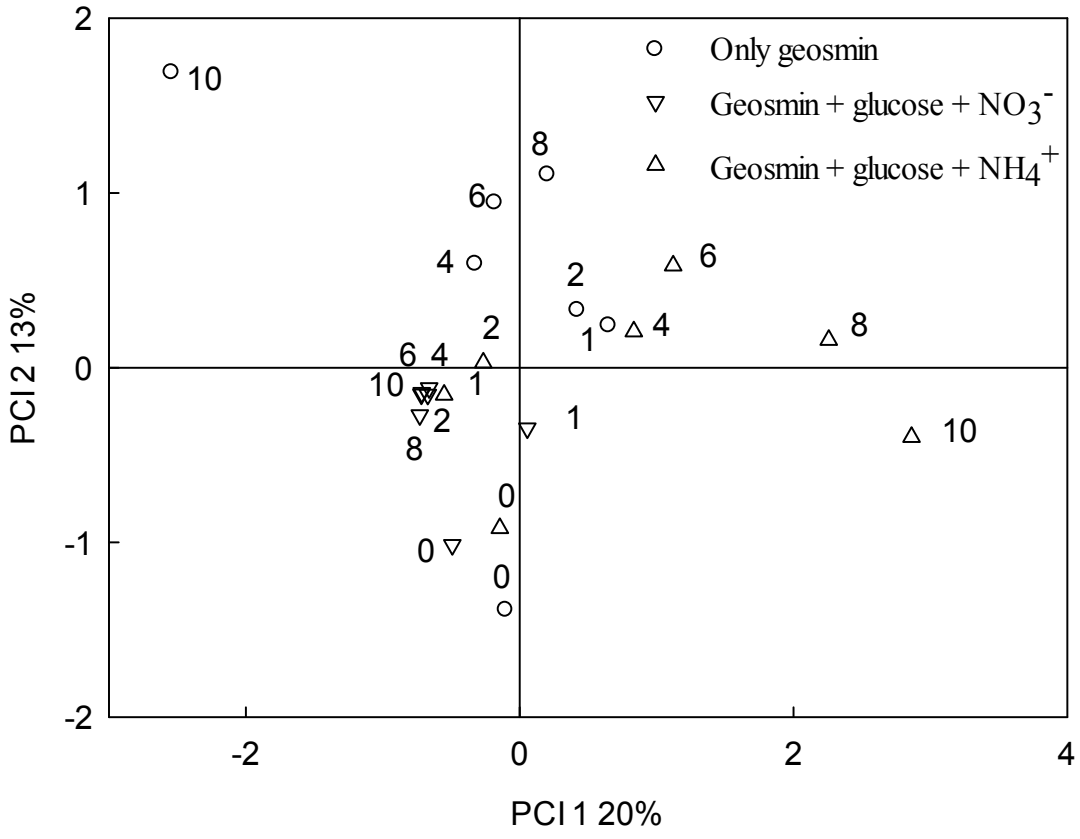


Fig. 3.10 Principal component analysis of the relative quantity generated by lane profiling for DGGE based on 16S rDNA. PCI 1 represents the first principal component explaining 20% characteristic of samples. PCI 2 represents the second principal component explaining 13% characteristic of samples. PCI 1 and PCI 2 are uncorrelated. The number of 0, 1, 2, 4, 6, 8 and 10 is incubation day. ○: Only geosmin; biofilm and geosmin, ▽: Geosmin + glucose + NO_3^-; biofilm with geosmin, glucose and nitrate, Δ: Geosmin + glucose + NH_4^+; biofilm with geosmin, glucose and ammonia.

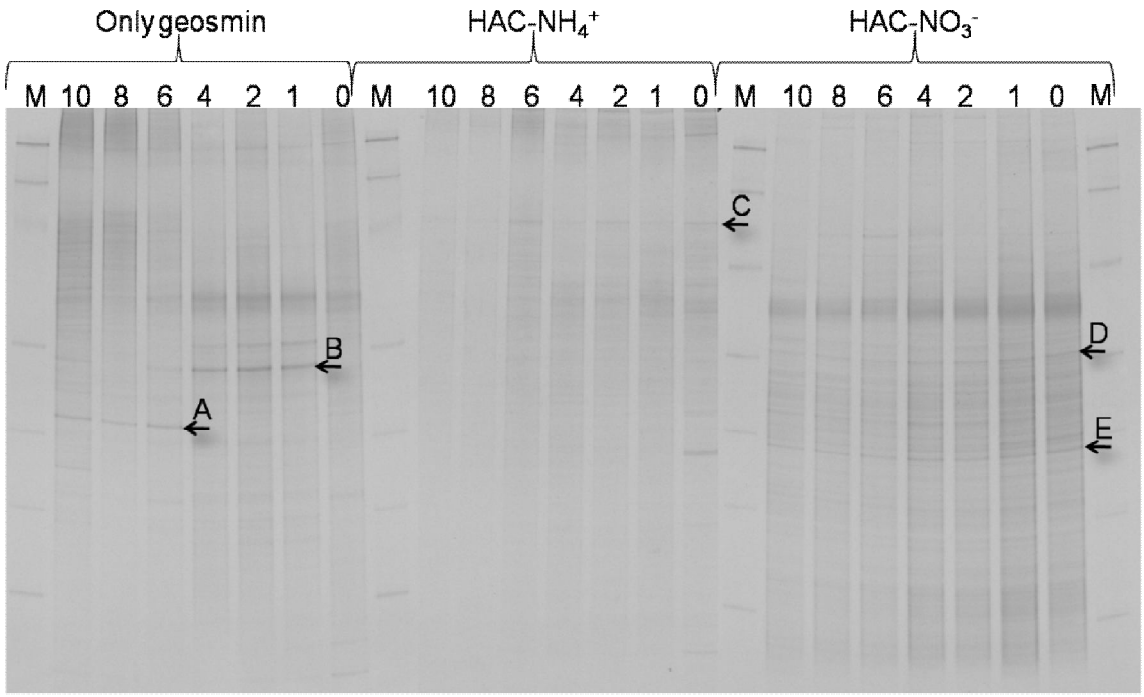


Fig. 3.11 DGGE band profiles of bacterial 16S rDNA fragments during geosmin degradation by microorganisms in the biofilm under only geosmin, HAC-NO_3^- and HAC-NH_4^+ conditions. 0-10 presents incubation day. M presents DGGE marker.

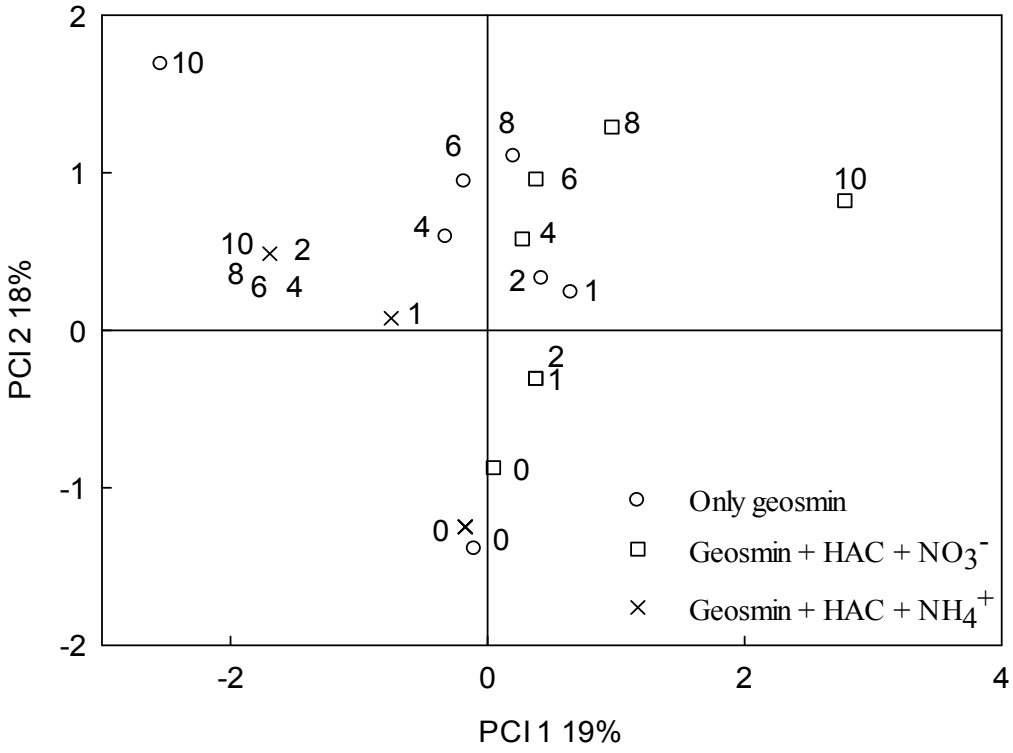


Fig. 3.12 Principal component analysis of the relative quantity generated by lane profiling for DGGE based on 16S rDNA. PCI 1 represents the first principal component explaining 19% characteristic of samples. PCI 2 represents the second principal component explaining 18% characteristic of samples. PCI 1 and PCI 2 are uncorrelated. The number of 0, 1, 2, 4, 6, 8 and 10 is incubation day. ○: Only geosmin; biofilm and geosmin, □: Geosmin + HAC + NO_3^-; biofilm with geosmin, HAC and ammonia, ×: Geosmin + HAC + NH_4^+; biofilm with geosmin, HAC and ammonia.

Chapter 4 Conclusions and future plan

4.1 Conclusions

For heterotrophic bacteria, organic carbon sources and inorganic nutrient substance (nitrogen and phosphorus) were important for bacteria growth. And also studies about geosmin degradation reported that geosmin degradation was influenced by natural organic matter when it is present at mg L^{-1}. However, the effect of organic carbon and inorganic nutrient substances (presented at μg L^{-1}) on geosmin biodegradation by biofilm is not clear. So this thesis aimed to investigate effect of organic carbon sources, inorganic nutrient substances on geosmin degradation and which is main factor effect geosmin biodegradation.

Effect of geosmin degradation experiment was done as following: glucose, sodium acetate and acetic acid was used as organic carbon source, which was added into the culture which was consisted of MSM medium, geosmin (500 ng L^{-1}) and biofilm. The bacterial number, activity and community composition were investigated by qPCR of 16S rDNA, ATP concentration and DGGE fingerprint, respectively. The results showed that geosmin degradation was a pseudo-first-order reaction when carbon occurred at μg L^{-1}. Glucose and NaAc stimulated geosmin degradation through increasing bacterial activity. HAC inhibited geosmin degradation. Glucose had significant stimulation effect on geosmin degradation. Carbon source also induced bacterial community composition change.

Effect of inorganic nutrients (nitrate, ammonia and phosphate), dual nutrients

substance (glucose-NO_3^-, glucose-NOH_4^+, HAC- NO_3^- and HAC- NOH_4^+) on geosmin degradation experiment was done as followings. These additives were spiked into flasks, which containing ultra pure water, geosmin (500 ng L^{-1}) and biofilm. The bacterial number, activity and community composition were studied by cell number (DAPI stain), ATP concentration and DGGE fingerprint, respectively. Results showed that nitrate, ammonia, phosphate, glucose-NO_3^- and glucose-NOH_4^+ also stimulated geosmin degradation through enzymes or cofactor. However, the influence of HAC-NO_3^- and HAC-NOH_4^+ on geosmin biodegradation was not significant. The phosphate had most significant effect on stimulating geosmin biodegradation, and organic carbon source was main factor affect geosmin biodegradation. Bacterial community composition changed depending on additives.

These results showed that geosmin degradation was stimulated by carbon sources (except for HAC) and nutrient which were the necessary component for bacterial growth. Carbon source and nutrient stimulated geosmin degradation by enzymes or cofactor produced by bacteria utilizing carbon source or nutrient. The stimulation of glucose was most significant. Bacterial community structure in all conditions changed which implied that geosmin could be degraded through many kinds of enzymes produced by different bacteria species.

4.2 Future plan

This study supplies an insight to geosmin degradation pathway. To date, geosmin degradation pathway is not clear, might be because geosmin degradation enzyme was

not special. So base on co-metabolism conception geosmin degradation pathway would be found, as phenol biodegradation is also co-metabolism.

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Acknowledgements

I would like to thanks to my thesis supervisor, Professor Sugiura Norio, from my heart depth. This thesis would not have been finished without his constant support and careful recommendations. And I greatly appreciated for all his help in life throughout my study in University of Tsukuba.

I especially express my thanks to Professor Utsumi Motoo, who taught me how to design experiment and write science paper. Although there were grief and happiness, I grew in these three years.

I am thankful to Professor Wang Guangcai, Dr. Shimizu Kazuya, and Dr. Li Jieming, Professor Zhang Zhenya, Professor Yang Yingnan and Professor Lei Zhongfang for their valuable advices and assistance to this study. I wish to express my gratitude to the following people for their helps and collaborations, Dr. Chie Amano, Mr. Shintaro Yamaguchi, Ms. Hirokawa Mariko and Ms. Wang Xin. I would like to thank all lab members who had worked with me and helped me when I had trouble. For this help each other atmosphere, I spend three happy years.

I would like to extend my thanks and appreciation to China Scholarship Council for giving me this chance to study in Doctoral Program of Graduate School of Life and Environmental Science, University of Tsukuba.

I would like to thanks my family for courage and enlightening me, when I encountered problems in study and life.