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Change in eubacteria in sludge by the addition of iron ion releasers

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【Abstract】

Reportedly the properties of sludge have been changed by installing iron ion releasers (also known as Σ Balls) in the bottom layer in the estuary of a river to supply ferrous iron ion into seawater. This study compared the time course between a tank with Σ Balls in sludge and a tank with sludge only. The emergent eubacteria were analyzed. As a result, in Tank A (sludge only), bacterial clones closely related to *Holophaga* were detected at a high frequency (number of clones = 5, homology = 82.3–86.3%), and in Tank B (with Σ Balls in sludge), bacterial clones closely related to *Chthoniobacter* (number of clones = 9, homology = 83.6–88.0%), and *Candidatus Magnetobacterium* (number of clones = 6, homology = 88.0–89.6%) were detected at high frequency.

【Key words】

Σ Balls, Sludge, Eubacteria, *Holophaga*, *Chthoniobacter*, *Magnetobacterium*

I Introduction

Sludge is soft mud that is present at the bottom of bodies of water such as slow rivers, canals, ports, and harbors. It contains specified hazardous substances, affects the human body via fishery products, contains much organic matter to release toxic gases, degrades the habitat of fish and shellfish, causes the emergence of fish with abnormal odor, and inhibits the function of ports by excessive accumulation¹⁾. To avoid such harmful effects, sediment improvement testing such as disseminating lime²⁾, cover sand³⁾, oyster shell⁴⁾, or clay⁵⁾, and oxygen supply⁶⁾, and the removal of sludge by dredging⁷⁾ have been conducted.

Reportedly the properties of sludge have been changed by installing iron ion releasers in the bottom layer in the estuary of a river to supply ferrous iron ion into seawater. The iron ion releasers are also known as Σ Balls invented by Mikio Sugimoto in Ube-city, Yamaguchi prefecture, west Japan⁸⁾.

However, the level and the mechanism of the change in sludge properties by iron ion releasers remain unknown. This study compared the time course between a tank with Σ Balls in sludge and a tank with sludge only. The emergent eubacteria

were analyzed.

II Materials and methods

In two 60-cm tanks A and B installed from April 10, 2009 to September 16, 2010, 7000 g of sludge was collected from the bottom layer in mouth of Eno river. Eno river flows in Nagato city, Yamaguchi prefecture (Figure 1), 47 L of dechlorinated tap water, and 8-10cm long large-flowered waterweeds, 10 g in total, collected from upstream of the same river, were added.

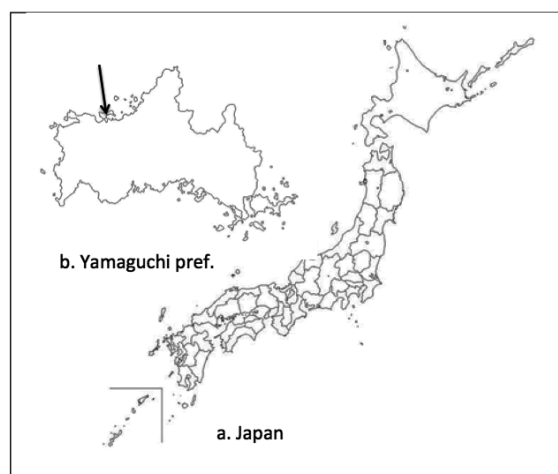


Figure 1 Sampling site of sludge (arrow line). The sludge was collected from the bottom layer in mouth of Eno river that flows in Nagato city, Yamaguchi prefecture (b.), Japan (a.).

Three Σ Balls were added only to tank B.

The time course of the temperature in Nagato city, Yamaguchi prefecture, during the test period is shown in the Figure 2.

In September 16, 2010, sediment was collected from the bottom layers of tanks A and B, and was analyzed for eubacteria clones.

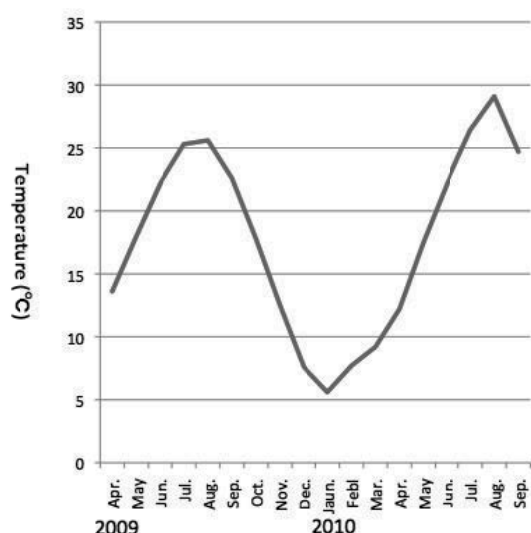


Figure 2. Monthly change of Temperature (°C) in Nagato-City, Yamaguchi-Pref. from April 2009 to September 2010.

Eubacteria clone analysis was conducted on November 15, 2010, at J-Bio 21 Corporation using ExtrapSoilDNAKitPlusVer.2 (J-Bio21) to extract and purify DNA from the samples. The DNA concentration in the DNA solution was measured using PicoGreen dsDNA Assay Kit (Invitrogen Corp).

III Results

Table 1 presents results from DNA concentration measurements.

Table 1 The results of density measurements of DNA

Sample name	Amount of sample (g)	Liquid measure of DNA after purified (μ /L)	Density of DNA after purified(μ /L)
Bottom sludge	0.5	100	24.1
Iron + Bottom sludge	0.5	100	11.5

With these purified DNA as templates, 16SrRNA gene from eubacteria was PCR-amplified using a primer(Table2^{9),10}).

Table2 Information of PCR primer name and base length and sequences

	Name	Base length(mer)	Sequence(5' -3')
Specific universal primer of eubacterias	27f	20	AGAGTTTGATCMTGGCTCAG
	Bcc1392 R	15	ACGGGCGGTGTGTAC

The number of PCR cycles was determined by conducting QPrimerPCR in advance to monitor the amplification process. The formation of PCR bias and artifacts can be reduced by stopping PCR reaction at an earlier stage.

However, if the reaction is ended too early, then the PCR product sufficient for subsequent analysis cannot be obtained¹¹). Therefore the optimal number of cycles to obtain the sufficient product for cloning was determined based on the monitoring result obtained using QPrimerPCR (Table 3).

Table 3 The results of number of PCR cycles using of liquid concentrate of DNA

Sample NAME	number of PCR cycles
Bottom sludge	21
Iron + Bottom sludge	21

The PCR amplification products of eubacteria 16SrDNA obtained as described above were cloned. They were subjected to sequence analysis for 96 clones each. The primer used for sequence analysis was 27f in Table 2, to read the nucleotide sequence of 16SrDNA. The resulting DNA sequences were matched with a public database to search for homology (BLAST search). Closely related bacterial species were predicted for each clone. Additionally, it is possible that clones with low homology can be derived from bacteria of new genus that differs from the genus that was presumed to be closely related.

Tank A (sludge only)

Nucleotide sequence information was obtained for 90 clones of 96 clones analyzed(Table 4). However, 12 clones among these were from chloroplasts of eukaryotes. Bacterial clones closely related to *Holophaga foetida* were detected at a high frequency (number of clones = 5, homology = 82.3–86.3%). Homology of not less than 96%,

which indicates the possibility that it is in the same genus of the closely related microorganism, was observed in 25 clones of 90 clones. Homology of not less than 99.7%, which reflects the possibility that it is in the same species of the closely related microorganism, was observed in 1 clone of 90 clones.

Tank B (with Σ Balls in sludge)

Nucleotide sequence information was obtained for 96 clones of 96 clones analyzed (Table 5). However, 1 clone among these was from chloroplasts of eukaryotes. Bacterial clones closely related to *Chthoniobacter* (number of clones = 9, homology = 83.6–88.0%), and *Candidatus Magnetobacterium* (number of clones = 6, homology = 88.0–89.6%) were detected at high frequency. Homology of not less than 96%, which indicates the possibility that it is in the same genus of the closely related microorganism, was observed in 12 clones of 96 clones. Homology of not less than 99.7%, which indicates the possibility that it is in the same species of the closely related microorganism, was observed in 0 clone of 96 clones. Nucleotide homology of not more than 90% to known microorganisms was detected in 55 clones.

IV Discussion

Bacterial clones closely related to *Holophaga* were detected at high frequency (number of clones = 5, homology = 82.3–86.3%) in tank A with sludge only. *Holophaga foetida* are known for the genus *Holophaga*. They grow in the presence of trimethoxybenzoate or syringate and produce sulfur compounds dimethyl sulfide and methanethiol¹²⁾. Both dimethyl sulfide and methanethiol produces foul odors even at a very low concentrations, thereby these are designated as specified offensive odor substances by the Offensive Odor Control Law and the Order for Enforcement of the Offensive Odor Control Law¹³⁾ Dimethyl sulfide at a high concentration irritates eyes and skin, and causes oxygen deficiency at a very high concentration. It

is also inflammable, causes fire/explosion hazards by reacting with oxidizers, and its mixture gas with air is explosive¹³⁾ It also is involved in the sulfur circulation on a global scale because it is highly volatile when it is generated in large quantities, and is presumed to affect climate change Methanethiol, which is produced by the decomposition of organic compounds in swamps and other water bodies, is contained in coal tar, crude oil, and natural gas in some regions¹³⁾.

In tank B with sludge and Σ Balls, bacterial clones closely related to *Chthoniobacter* (number of clones = 9, homology = 83.6–88.0%), and those closely related to *Magnetobacterium* (number of clones = 6, homology = 88.0–89.6%) were detected at a high frequency.

Genus *Chthoniobacter* belongs to phylum *Verrucomicrobia*. It is known as methane-oxidizing, with a function to suppress the release of methane gas from soil into the air¹⁴⁾. Genus *Magnetobacterium* are magnetic bacteria observed in freshwater and ocean soil, and is presumed to have magnetosomes abundant in iron in the cell. Their emergence is dependent on the presence or absence of oxidizing–reducing substances and sulfur-reducing organisms^{15),16)}. It also is said that the presence of dissolved iron is the requirement for their survival¹⁷⁾.

Magnetic bacteria under certain circumstances produce iron sulfide in particular. Their inhabitation in the area with no oxygen has been confirmed. They are found only rarely in water or soil where there is abundant oxygen. It was confirmed that Fe₃S₄, FeS₂, and Fe_{1-x}S are present as magnetosomes in the cell¹⁸⁾. Whereas magnetic bacteria conduct microaerobic oxygen respiration to obtain energy necessary for their growth, they also simultaneously express enzymes of the denitrification pathway, which is an anaerobic respiratory pathway¹⁹⁾. The facts described above suggest that *Holophaga* that produced methyl sulfide and methanethiol with foul odors emerged in the case of sludge only, but their

emergence might be suppressed by adding Σ Balls into sludge.

By the addition of ferrous iron ion releaser into sludge, eubacteria were detected, which were closely related to *Chthoniobacter* that prevented the release of methane gas, a greenhouse gas, as well as to *Magnetobacterium*, which cleaned up hydrogen sulfide sediment.

The addition of iron ion releasers might change the eubacterial flora and improve the environment in sediment at the bottom of a river. Future studies should be undertaken for detailed examination of which component in sludge is affected by iron ion releasers and how the eubacterial flora is influenced by analyzing sludge components and isolating eubacteria.

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Table 4 Classified information of closely-related microorganisms in Tank A (sludge only). The each number indicate the number of clones

Domain	Phylum	Class	Order	Family	Genus	Species																			
Proteobacteria	Bacteria	36				Comamonadaceae	2	Schlegelella	1	Caenibacterium sp. HY10(2010)	1														
										Limnohabitans	1	Limnohabitans parvus	1												
						Burkholderiales	7			Methylibium	2	Methylibium sp. T2-YC6780	1												
										Methylibium fulvum	1		1												
										Thiomonas	1	Thiomonas sp. 6C	1												
										Thiobacter	1	Thiobacter subterraneus	1												
						Betaproteobacteria	11			Aquabacterium	1	Aquabacterium sp. AKB-2008-KU6	1												
						Rhodocyclales	3	Rhodocyclaceae	3	Dechloromonas	2	Dechloromonas sp. MissR	2												
										Propionivibrio	1	Propionivibrio limicola	1												
						Hydrogenophilales	1	Hydrogenophilaceae	1	Thiobacillus	1	Thiobacillus thioparus	1												
						Desulfuromonadales	4	Desulfuromonadaceae	2	Desulfuromonas	2	Desulfuromonas thiophila	1												
												Desulfuromonas michiganensis	1												
										Pelobacteraceae	1	Pelobacter	1	Pelobacter sp. A3b3	1										
										Geobacteraceae	1	Geobacter	1	Geobacter pelophilus	1										
						Deltaproteobacteria	10	Desulfobacteriales	3	Desulfobacteraceae	3	Desulfonema	1	Desulfonema magnum	1										
												Desulfobacterium	1	Desulfobacterium anilini	1										
												Desulfatibacillum	1	Desulfatibacillum aliphaticivorans	1										
						Myxococcales	2	Polyangiaceae	1	Chondromyces	1	Chondromyces apiculatus	1												
								Phaselicystidaceae	1	Phaselicystis	1	Phaselicystis flava	1												
						Syntrophobacteriales	1	Syntrophobacteraceae	1	Syntrophobacter	1	Syntrophobacter pfennigii	1												
						Rhizobiales	5	Bradyrhizobiaceae	2	Hyphomicrobiaceae	2	Hyphomicrobium	2	Hyphomicrobium vulgare	1										
												Hyphomicrobium sp. 16-60	1												
										Nitrobacter	1	Nitrobacter hamburgensis	1												
										Balneimonas	1	Balneimonas sp. SWF67558	1												
						Alphaproteobacteria	10	Beijerinckiaceae	1	Chelatococcus	1	Chelatococcus sp. P-117	1												
												Catellibacterium sp. dv4	1												
												Catellibacterium sp. A1-9	1												
												Rhodobacter	1	Rhodobacter sp. TCRI	1										
						Sphingomonadales	1	Sphingomonadaceae	1	Sphingomonas	1	Sphingomonas sp. SAL_12	1												
						Caulobacteriales	1	Caulobacteraceae	1	Caulobacter	1	Caulobacter sp. H62	1												
Pseudomonadales	2	Pseudomonadaceae	2	Rhizobacter	1	Rhizobacter sp. Z0-YC6816	1																		
				Pseudomonas	1	Pseudomonas sp. Z49zhy	1																		
Gammaproteobacteria	5	Xanthomonadales	1	Xanthomonadaceae	1	Lysobacter	1	Lysobacter sp. T-15	1																
				Methylococcales	1	Methylococcaceae	1	Methylomicrobium	1	Methylomicrobium japonense	1														
				Alteromonadales	1	Alteromonadaceae	1	Microbulbifer	1	Microbulbifer sp. SPO729	1														
Bacteria	78					Holophaga	5	Holophaga foetida	5																
						Acidobacteria	7	Holophagae	7	Holophagales	7	Holophagaceae	7	Geothrix	2	Geothrix fermentans	2								
														Verrucomicrobia	6	Verrucomicrobiae	4	Verrucomicrobiales	4	Verrucomicrobiaceae	4	Rubritalea	1	Rubritalea sabuli	1
																						Roseibacillus	1	Roseibacillus persicicus	1
						Prostheobacter	1	Prostheobacter dejongeii	1																
						Luteolibacter	1	Luteolibacter pohmpiensis	1																
								Spartobacteria	1	Chthoniobacter	1	Chthoniobacter flavus	1												
								Methylacidiphilales	1	Methylacidiphilaceae	1	Methylacidiphilum	1	Methylacidiphilum fumarolicum	1										
						Chloroflexi	6	Anaerolineae	5	Anaerolineales	5	Anaerolineaceae	5	Levilinea	2	Levilinea saccharolytica	2								
														Longilinea	1	Longilinea arvoryzae	1								
														Bellilinea	1	Bellilinea caldijisuluae	1								
								Dehalococcoidetes	1	Dehalococcoides	1	Dehalococcoides sp. BH180-15	1												
						Actinobacteria	6	Actinobacteria	6	Actinomycetales	3	Streptomycetaceae	1	Streptomyces	1	Streptomyces rochei	1								
												Mycobacteriaceae	1	Mycobacterium	1	Mycobacterium sp. CNJ879 PL04	1								
												Micromonosporaceae	1	Actinoplanes	1	Actinoplanes penicillatus	1								
												Acidimicrobiales	2	Acidimicrobiaceae	2	Acidithiobacterium	2	Acidithiobacterium sp. P1	2						
								Solirubrobacteriales	1	Solirubrobacteraceae	1	Solirubrobacter	1	Solirubrobacter sp. BXN5-15	1										
						Flavobacteria	2	Flavobacteriales	2	Flavobacteriaceae	2	Flavobacterium	1	Flavobacterium sp. 01xTSA06A D03	1										
												Bergeyella	1	Bergeyella sp. M2T8B3	1										
						Bacteroidetes	5	Cytophagia	2	Cytophagales	2	Cytophagaceae	2	Cytophaga	2	Cytophaga sp. Dex80-37	1								
														Cytophaga fermentans	1										
								Bacteroidia	1	Bacteroidales	1	Marinilabilia	1	Marinilabilia salmonicolor	1										
						Firmicutes	4	Clostridia	2	Clostridiales	2	Ruminococcaceae	1	Acetivibrio	1	Acetivibrio cellulolyticus	1								
												Clostridiaceae	1	Caloramator	1	Caloramator australicus	1								
												Erysipelotrichi	1	Erysipelotrichales	1	Erysipelotrichaceae	1	Solobacterium	1	Solobacterium moorei	1				
												Bacilli	1	Bacillales	1	Bacillaceae	1	Bacillus	1	Bacillus oshimensis	1				
						Spirochaetes	3	Spirochaetes	3	Spirochaetales	3	Spirochaetaceae	3	Spirochaeta	3	Spirochaeta sp.	1								
																Spirochaeta bajacaliforniensis	1								
																Spirochaeta caldaria	1								
						Chlorobi	2	Ignavibacteria	2	Ignavibacteriales	2	Ignavibacteriaceae	2	Ignavibacterium	2	Ignavibacterium album	2								
						Thermotogae	1	Thermotogae	1	Thermotogales	1	Thermotogaceae	1	Thermotoga	1	Thermotoga thermarum	1								
						Planctomycetes	1	Planctomycetacia	1	Planctomycetales	1	Planctomycetaceae	1	Pirellula	1	Pirellula staleyi	1								
						Gemmatimonadetes	1	Gemmatimonadetes	1	Gemmatimonadales	1	Gemmatimonadaceae	1	Gemmatimonas	1	Gemmatimonas aurantiaca	1								
						Eukaryota	12	Bacillariophyta	11	Fragilariophyceae	10	Fragilariales	10	Fragilariaceae	10	Synedra	6	Synedra fragilaroides	6						
Nanofrustulum	3	Nanofrustulum shiloi	3																						
Fragilaria	1	Fragilaria pinnata	1																						
Bacillariophyceae	1	Bacillariaceae	1	Cylindrotheca	1											Cylindrotheca closterium	1								
Chlorophyta	1	Chlorophyceae	1	Sphaeropleales	1	Scenedesmeceae	1	Scenedesmus	1	Scenedesmus obliquus	1														

Table 5 Classified information of closely-related microorganisms in Tank B (with Σ balls in sludge). The each number indicate the number of clones.

Domain	Phylum	Class	Order	Family	Genus	Species				
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus	<i>Syntrophus</i> sp.	2			
					Smithella	<i>Smithella propionica</i>	2			
					8					
				Syntrophorhabdaceae	Syntrophorhabdus	<i>Syntrophorhabdus aromaticivorans</i>	3			
				3						
				Syntrophobacteraceae	Syntrophobacter	<i>Syntrophobacter wolnii</i>	1			
				1						
				Desulfobacterales	Desulfobacteraceae	Desulfonema	<i>Desulfonema magnum</i>	4		
						6				
						Desulfosarcina	<i>Desulfosarcina</i> sp. SD1	1		
		Desulfatibacillum	<i>Desulfatibacillum alkenivorans</i>		1					
		Desulfobulbaceae	Desulforhopalus		<i>Desulforhopalus singaporensis</i>	1				
			Desulfobulbus		<i>Desulfobulbus</i> sp. RPJ35L17	1				
		2								
		Desulfuromonadales	Geobacteraceae		Geoalkalibacter	<i>Geoalkalibacter subterraneus</i>	2			
					4					
			Geopsychrobacter		<i>Geopsychrobacter electrodiphilus</i>	1				
			Geobacter	<i>Geobacter</i> sp. G02	1					
			Pelobacteraceae	Pelobacter	<i>Pelobacter carbinolicus</i>	1				
		Myxococcales	Myxococcaceae	Anaeromyxobacter	<i>Anaeromyxobacter</i> sp. Fw109-5	1				
			Haliangiaceae	Haliangium	<i>Haliangium tepidum</i>	1				
		2								
		Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	<i>Burkholderia nodosa</i>	2			
					3					
				Cupriavidus	<i>Cupriavidus pinatubonensis</i>	1				
			Alcaligenaceae	Derxia	<i>Derxia gummosa</i>	1				
			7							
			Rhodocyclales	Rhodocyclaceae	Thiobacter	<i>Thiobacter subterraneus</i>	1			
					Sphaerotilus	<i>Sphaerotilus hippel</i>	1			
			2							
			Gallionellales	Gallionellaceae	Aquabacterium	<i>Aquabacterium</i> sp. P-113	1			
					Uliginosibacterium	<i>Uliginosibacterium gangwonense</i>	1			
		2								
		Gammaproteobacteria	Methylococcales	Methylococcaceae	Methylocaldum	<i>Methylocaldum</i> sp. E10a	2			
					5					
			Methylobacter	<i>Methylobacter tundripaludum</i>	2					
			3							
			Alteromonadales	Alteromonadaceae	Haltea	<i>Haltea</i> sp. SY02	1			
					Haltea	<i>Haltea salixigens</i>	1			
			2							
			Chromatiales	Chromatiaceae	Halochromatium	<i>Halochromatium</i> sp. MTK6IM08	1			
					1					
			Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylosinus	<i>Methylosinus</i> sp. 24-21	2		
		3								
		Methylocystis			<i>Methylocystis</i> sp. KS7	1				
Phyllobacteriaceae	Phyllobacterium	<i>Phyllobacterium myrsinacearum</i>	1							
4										
Verrucomicrobia	Spartobacteria	Spartobacterales	Spartobacteriaceae	Chthoniobacter	<i>Chthoniobacter flavus</i>	9				
				9						
				Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Bellilinea	<i>Bellilinea caldifistulae</i>	3
								8		
								Leptolinea	<i>Leptolinea tardivitalis</i>	2
								Anaerolinea	<i>Anaerolinea thermolimosana</i>	2
					9					
					Dehalococcoidetes	Dehalococcoidales	Dehalococcoidaceae	Levilinea	<i>Levilinea saccharolytica</i>	1
								Dehalococcoides	<i>Dehalococcoides</i> sp. BH180-15	1
					1					
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae		Candidatus Magnetobacterium	<i>Candidatus Magnetobacterium</i> bc	6			
					7					
				Nitrospira	<i>Candidatus Nitrospira bockiana</i>	1				
				7						
				Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Catabacter	<i>Catabacter</i> sp. YIT 12065	2
								2		
								Desulfobacterium	<i>Desulfobacterium hafniense</i>	1
								Gracilibacter	<i>Gracilibacter thermotolerans</i>	1
					Lutispora	<i>Lutispora thermophila</i>	1			
				6						
Thermolithobacteria	<i>Thermolithobacter ferrireducens</i>	1								
1										
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Cytophaga	<i>Cytophaga</i> sp. Dex80-37	3				
				5						
Meniscus	<i>Meniscus glaucopsis</i>	2								
5										
Planctomycetes	Phycisphaerae	Phycisphaerales	Phycisphaeraceae	Phycisphaera	<i>Phycisphaera mikrensis</i>	2				
				3						
Pirellula	<i>Pirellula staleyi</i>	1								
3										
Acidobacteria	Holophagae	Holophagales	Holophagaceae	Candidatus Solibacter	<i>Candidatus Solibacter usitatus</i>	1				
				3						
Anabaena	<i>Anabaena</i> sp. A277	1								
Anabaena	<i>Anabaena</i> sp. 08-05	1								
2										
Chlorobi	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	Ignavibacterium	<i>Ignavibacterium album</i>	2				
				2						
Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	Acidimicrobium	<i>Acidimicrobium ferrooxidans</i>	1				
				2						
Coriobacterium	<i>Coriobacterium parvulum</i>	1								
2										
Spirochaetes	<i>Spirochaeta bajacaliforniensis</i>	1								
1										
Fusobacteria	<i>Fusobacterium varium</i>	1								
1										
Eukaryota	Streptophyta	Coleochaetophyceae	Coleochaetales	Coleochaetaceae	Coleochaete	<i>Coleochaete orbicularis</i>	1			