Community structure and ecological role of anaerobic protists in UASB reactor treating domestic sewage

都市下水処理 UASB 槽における嫌気性原生動物の群集構造解析と生態学的役割に関する研究

Doctoral thesis by Yuga Hirakata

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Doctor of Engineering

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Chapter I

General introduction

1.1 Bacground and objective

Anaerobic treatment technology such as up-flow anaerobic sludge blanket (UASB) system has been widely applied for high- and medium- strength industrial wastewater treatment because of no need aeration, less excess sludge production, and energy recovery as methane (Rajeshwari et al., 2000; Frankin, 2001). Furthermore, the UASB has been of great interest to developing countries for domestic sewerage treatment because it has the following advantages against conventional aerobic activated sludge systems; cheaper operational costs and high performance stability even when stable power supply is not available (Syutsubo et al., 2011). Although UASB system has been applying to domestic sewage treatment in both tropical and subtropical regions, including India and Brazil (Sato et al., 2007; Heffernan et al., 2011; Florencio et al., 2001), this system has not applied to domestic sewage treatment in cooler region such as Japan. This is due to low temperature and characteristic of domestic sewage.

Domestic sewage is characterized by low chemical oxygen demand (COD) concentrations (100–600 mg/L), high fractions of suspended solids (SS) components such as cellulose and protein (50–60% of the total COD). Because application of anaerobic treatment processes at low temperatures (lower than 20 °C) requires a longer sludge retention time (SRT) (de Man et al. 1986), the accumulation of SS inside the UASB reactor treating domestic sewage can occur and lead to washout of active biomass. Thus, these conditions lead to a decrease in methanogenic activity and process performance (Zeeman and Lettinga 1999). In addition, anaerobic microbial activities are very low at low temperature (Uemura and Harada, 2000). Although previous study had applied pilot-scale UASB systems for domestic sewage in japan, cellulose accumulation occurred at also lower temperature season (Syutsubo et al., 2011). Takahashi et al. (2011) reported that *Ruminococcus*-related bacteria species as a cellulose decomposer was detected in UASB reactor treating domestic sewage by 16S rRNA gene analysis, but accumulation of cellulose has occurred at condition of longer SRT and low temperature. Therefore, research on organic matter removal by sulfate reducing bacteria instead of methanogen under psychrophilic conditions and improvement of anaerobically treated effluent quality using aerobic post treatment systems have been advanced (Sumino et al., 2007; Takahashi et al., 2011).

In an UASB pilot plant treating domestic sewage, anaerobic bacteria, archaea and also protozoa are coexisting (Fig. 1-1). In anaerobic wastewater treatment processes, a complex community consisting of many interacting microbial species degrades organic compound such as carbohydrate, proteins, and lipids, in the absence of oxygen, into methane and CO_2 (Mao et al., 2015). However, although prokaryotes (bacteria and archaea) play major role in these processes, how much protist contributes to wastewater treatment in the UASB reactor was poorly understood. In contrast, protist are important component in both ecosystems and treatment performance in aerobic treatment processes like activated sludge. Protist is used as indicator of treatment performance in aerobic treatment processes because information about relationship between protists and operation condition was accumulated (Foissner,

2016). In aerobic treatment systems, protist and metazoa were predator of prokaryotes, protist was used with metazoa for decreasing sludge production from aerobic wastewater treatment plant (Lee and Welander, 1996; Ghyoot and Verstraete, 2000). In addition, predation by protist could not only reduce bacterial population but also stimulate bacterial activity in microbial ecosystems (Pussard, 1994; Mattison and Harayama, 2001). Furthermore, protist can feed particle organic matter include bacterial cell and contribute high COD removal efficiency and low effluent turbidity (Fenchel, 1980; Holubar et al., 2000).

Anaerobic protists are also predator of bacteria in anaerobic ecosystems and could ingest particle organic matter (Fenchel and Finlay, 1992; Narayanan et al., 2007). In addition, protist population correlated with COD removal and methane production in anaerobic continuous stirred tank reactors (CSTR) (Priya et a., 2007). Therefore, anaerobic protists in UASB reactor also may contribute and improve treatment performance such as SS and COD removal and reduction of excess sludge production. However, very limited information on protist in anaerobic wastewater treatment systems is available compared to aerobic wastewater treatment processes. In particular, function of protists in the UASB reactor treating domestic sewage has been not reported previously.

The study in this thesis has focused on anaerobic protist in the UASB reactor treating domestic sewage. Protists are important components of ecosystems in wastewater treatment processes. However, little is known about their function and community structure in anaerobic wastewater treatment systems, in particular, in an UASB reactor. Hence, the main objective of this doctoral thesis was to accumulate the fundamental knowledge about function and community structures of anaerobic protist in UASB reactor. For these purpose, we aimed to 1) characterize anaerobic protist community and its temporal

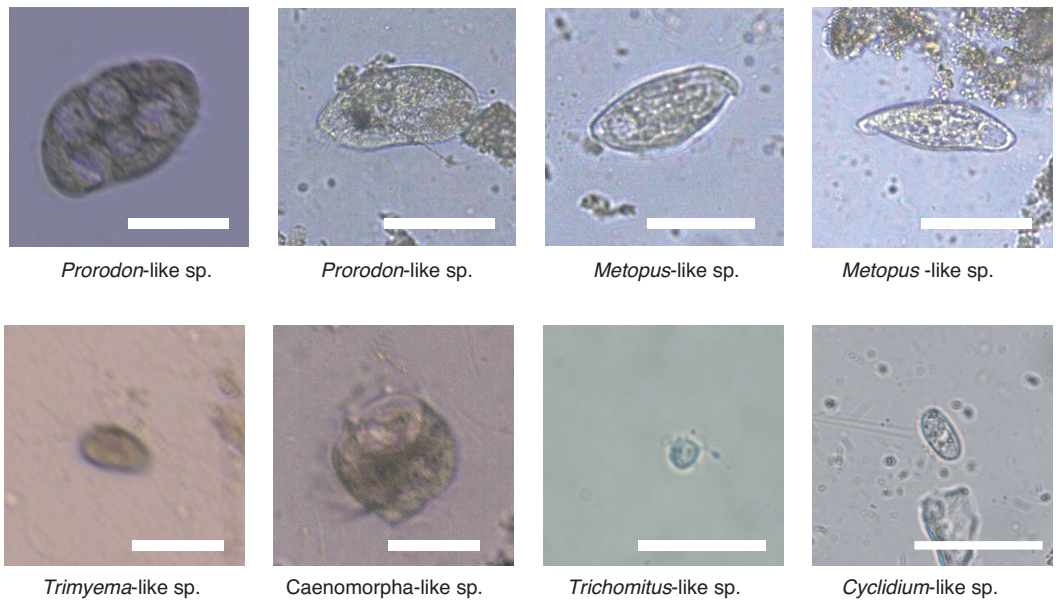

Figure 1-1. Anaerobic protists observed in UASB reactor treating domestic sewage. The scale bar is 50 μm.

variation, 2) isolate anaerobic protists and examine their physiological characteristics, and 3) investigate the influence of predation by protists on prokaryotic community function, structure, and diversity in UASB reactor treating domestic sewage.

1.2 Outline of this thesis

The thesis organized five chapters to achieve our objectives. The background, objectives and outline of this thesis are introdeucd in Chapter I. A literature review on anaerobic protist is provided in Chapter II. Application of 18S rRNA gene amplicon sequencing for protist community structure and analysis of its temporal variation in UASB reactor treating domestic sewage is described in Chapter III. In Chapter IV, Isolation of anaerobic protists and thier physiological characteristics such as ingestion rate, generation time, predation behavior and metabolite are described. Effect of predation by anaerobic protists on prokaryotic community function, structure, and diversity in the UASB reactor treating domestic sewage is described in Chapter V. Lastly, the summarized work and conclusions are discussed in Chapter VI.

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Chapter II

Literature review

2.1 Anaerobic protist

Anaerobic protists have been found in a wide range of anoxic ecosystems including anoxic freshwater (Massana and Pedrós-Alió, 1994; Bourland et al., 2014), marine sediments (Esteban et al., 1994; van Bruggen et al., 1986), landfill sites (Fenchel and Finlay., 1990; Finlay and Fenchel, 1991), rice field soil (Schwarz and Frenzel, 2005; Murase et al., 2006), rumen (Ivan et al., 2001; Ohene-Adjei et al., 2007), termite gut (Ohkuma et al., 2015; Kuwahara et al., 2017), and also wastewater treatment plant (Agrawal et al., 1997). Among anaerobic protists, parasite species of human and animal such as *Trichomonas* are also well known in field of parasitology (Dimasuay et al., 2013; Wen-Chao et al., 2018). Although parasite species absorbed nutrients by osmotrophy, most of free-living protists ingest bacteria for growth (Fenchel and Finlay, 1990). Additionally, anaerobic protists could ingest not only bacteria but also particle organic matter. Anaerobic ciliate *Metopus* sp. was cultivated axenically by using wheat powder as a substrate (Narayanan et al., 2007). Priya et al. (2007) reported that anaerobic ciliate could ingest directly particle organic matter from results of batch test fed with colloidal sodium oleate. Rumen protists such as *Diploplastron*, *Entodinium* and *Eudiplodinium* can degrade cellulose particle and starch grain directly and digest intracellularly (Coleman, 1992; Bełżecki et al., 2017; Czauderna et al., 2019).

As characteristics of anaerobic protists, they lack mitochondria and have a unique organelle, hydrogenosome instead of mitochondria, in which organic matter (i.e., pyruvate) is oxidized to hydrogen, carbon dioxide, and volatile fatty acids for ATP synthesis (Fig. 2-1) (Steinbuchel and Müller, 1986; Finlay and Fenchel, 1989; Mueller, 1993; Shinzato et al., 2007). The hydrogenosome could have evolved from mitochondria by adaptations under anaerobic conditions of ecological constraints (Germot et al., 1996; Boxma et al., 2005). Hence, hydrogenosome is found in various groups of fungi and protist, not limited to specific group (Müller et al., 2012; Zimorski et al., 2019). In addition to hydrogenosome, anaerobic organelle mitosome that are the most highly reduced forms of mitochondria, that do not produce ATP was also found in protist group such as Amoebozoa and Excavata and fungi of Microsporidia (Goldberg et al., 2008; Van Der Giezen et al., 2009). Among other than pritst and fungi, several groups of invertebrates also have reported to possess anaerobic mitochondria (Tielens et al., 2002; Hellemond et al., 2003).

Anaerobic protists in anoxic ecosystems harbor endosymbiotic prokaryotes (Fig. 2-1, Shinzato et al., 2018). Methanogen was most popular endosymbiont of free-living anaerobic protist (van Bruggen et al., 1983). In addition, *Metopus* ciliates host endosymbiotic methanogens affliated with the archaeal genera *Methanobacterium*, *Methanoplanus*, *Methanocorpusculum*, or *Methanosaeta* (Narayanan et al.,

2009; Embley et al., 1992a,b). *Trimyema* ciliates also have been reported to possess archaeal genera *Methanobrevibacter* or *Methanocorpusculum* as endosymbiont (Finlay et al., 1993; Shinzato et al., 2007). Therefore, anaerobic protist seemed to be able to construct symbiotic relationship with various methanogen speceis. These endosymbiotic methanogens convert hydrogen produced by anaerobic protist to methane gas (Müller, 1993). The elimination of endosymbiotic methanogens by the specific methanogen inhibitor, 2-bromoethanesulfornic acid (BES), was previously reported to result in a 30% reduction in the growth yield of *Metopus contortus* (Fenchel a nd Finlay, 1991). In addition to endosymbiotic methanogens, ectosymbiotic sulfate-reducing bacteria have been detected in *M. contortus*; however, the molecular phylogeny of these bacteria remains unknown (Fenchel and Ramsing, 1992). Endosymbiotic bacteria were often detected in anaerobic protist cells by fluorescence in situ hybridization analysis (Clarke et al., 1993). These Endosymbiotic bacteria are expected to contribute growth of host prtists although thier function remains unknown (Shinzato et al., 2007). In anaerobic wastewater treatment processess, anaerobic protists that possess endosymbiotic methanogen could degrade particle organic matter like bacterial cell to methane gas, suggesting contribution to sludge reduction and methane production.

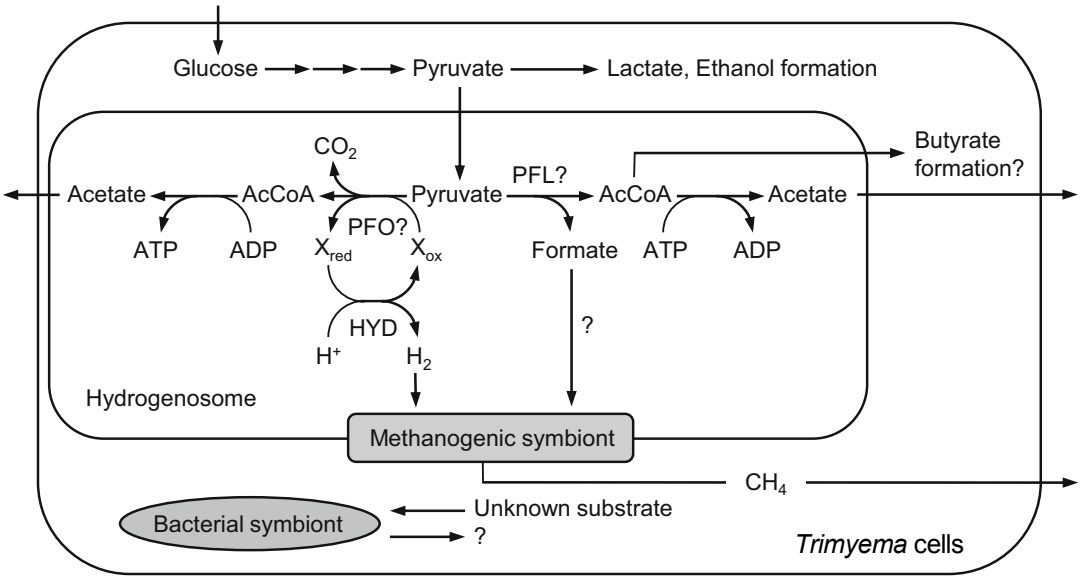

Figure 2-1. Speculative metabolic schemes of carbohydrate metabolism in the symbiotic consortium of *T. compressum*. Abbriviations: AcCoA, acetyl-CoA; HYD, hydrogenase; PFL, pyruvate formate lyase; PFO, pyruvate ferredoxin oxidoreductase. Xox, red, unknown electron carrier. Methanogenic symbionts are capable to use both hydrogen and formate as the substrate for methanogenesis. Substrate and contribution of bacterial symbionts are unknown (from Shinzato et al., 2018).

2.2 Protist observed in anaerobic wastewater treatment plant

Observation of *Metopus*-like free-living anaerobic ciliates in a high-rate anaerobic reactor that received low-strength wastewater by Agrawal et al. (1997) was first report about anaerobic protist in wastewater treatment plant. However, involvement of anaerobic protist in anaerobic treatment process was not investigated in their reports.

In anaerobic continuous stirred tank reactors (CSTR) fed with oleate and acetate under mesophilic condition (30 ± 2˚C), ciliate such as *Prorodon, Cyclidium, Metopus, Spathidium, Loxodes, Vorticella, Loxophyllum, Brachonella,* and *Discomorphella* and flagellates such as *Rhynchomonas, Naeglaria, Amoeboflagellates, Tetramitus, Trepomonas and Bodo, and Menoidium* have been observed microscopically (Priya et al., 2007; 2008). The population of ciliate was ranged 10^{3-4} cells mL^{-1} and flagellate was ranged 10^{4-6} cells mL^{-1} in CSTR. Among protist speceis, flagellates were observed during increased VFA concentration and affected periods of biomethanation. On the other hand, abundance of ciliates significantly correlated with the reduction of MLSS, higher COD removal and methane production Priya et al. (2008) also performed comparative experiment between batch culture fed with oleate and acetate and protist-inhibited culture that obtained by adding cycloheximide. In batch tests, increased COD removal and methane production was observed in sludge having ciliates as compared with sludge without protozoa.

In also anaerobic leach bed reactor (ALBR) for biomethanation of lignocellulose biomass, protist has been observed (Prabhakaran et al., 2016). In ALBR, the protist community in the digester composed of ciliates including *Metopus*, *Cyclidiumand,* and *Colpoda* and flagellates like *Menoidium*, *Rhyncomonas*, and *Bodo*. Protist abundance were ranged 10^{2-4} cells mL^{-1}, flagellate *Menoidium* was most dominant protist in ALBR. Methane production and hydrolytic enzyme activities, volatile fatty acid production, and biogas production in ALBR were correlated positively with ciliate and flagellate populations. These reports suggested that anaerobic protist may play important role in anaerobic wastewater treatment systems.

Previous studies have not observed anaerobic protists in anaerobic wastewater treatment system for high- and medium- strength industrial wastewater (Kuroda et al., 2016; Tran et al., 2017; Watari et al., 2017). Growth of anaerobic protist Metopus sp. was strongly inhibited by high acetate, butyrate, and propionate concentration (> 0.05 M) (Narayanan et al., 2007). Thus, anaerobic protists in anaerobic systems for industrial wastewater also could be inhibited by high VFA concentration resulted from high organic loading rate.

2.3 Role of anaerobic protists in anoxic ecosystems

Anaerobic protist is major predator in anoxic ecosystems, bacterial abundance was controlled by predation (Fenchel and Finlay, 1990). In sediment microbial fuel cells, predation by anaerobic protist greatly decreased anode biofilms and reduced current up to 91% (Holmes et al., 2015). On the other hands, anaerobic protist cannot control the bacteria in anoxic layer of lake because of both the low abundance and low feeding rate (Massana and Pedrós-Alió, 1994; Oikonomou et al., 2014). Thus, protist number needs to be maintained high concentration through long term for analyze effect of predation by protist on prokaryotes.

Anaerobic protists are known to be stimulated microbial activity in ecosystem. Biagini et al. (1998) reported that introduction of anaerobic ciliate *Metopus palaeformis* to anaerobic microcosms resulted in reduction of bacterial abundance but increase of methanogenic and sulfide reduction activities. *Metopus palaeformis* also has been reported to harbor endosymbiotic methanogen in inside of thier cells (Fenchel and Finlay, 1992; Embley et al., 1992), contributes to methane production. However, if the methanogenic endosymbionts in each ciliate assume to produce 0.37 pM CH_4 h^{-1} (Fenchel and Finlay, 1992), with an average number of 200 cells ml^{-1} over a period of 450 h, thier contribution was estimated very low (8 % of total methane production at best). Biagini et al. (1998) concluded that protist excretions such as organic acids (acetate and propionate) were most likely responsible for stimulation of microbial activity.

Endosymbiotic prokaryotes of anaeorbi protists have also known to play important role such as methane production. Julian Schwarz and Frenzel (2005) reported that nearly all methane produced from H_2/CO_2 could be attributed to endosymbiotic methanogen of anaerobic ciliate in rice field soil. This could be due to these endosymbiotic methanogens protected from the competition for substrates with other bacteria. This contribution to methane production by endosymbiotic methanogen of anaerobic protists was also observed in municipal landfill and subsurface sediments (Finlay and Fenchel, 1991; Holmes et al., 2014).

Prabhakaran et al. (2016) proposed a hypothetical scheme through which protist could increase methane gas in anaerobic reactors (Fig. 2-2). One possibility is that endosymbiotic methanogens can increase methane production. Second possibility is that extracellular hydrolytic enzymes from protist can enhance the breakdown of complex organics leading to increased VFA production that can subsequently enhance methanogenesis. Third possibility is that protists contirbute to hydrolyze particulate organic matter, and released soluble organics released by the grazing fauna can contribute to the pool of substrate like organic acids for methanogenesis.

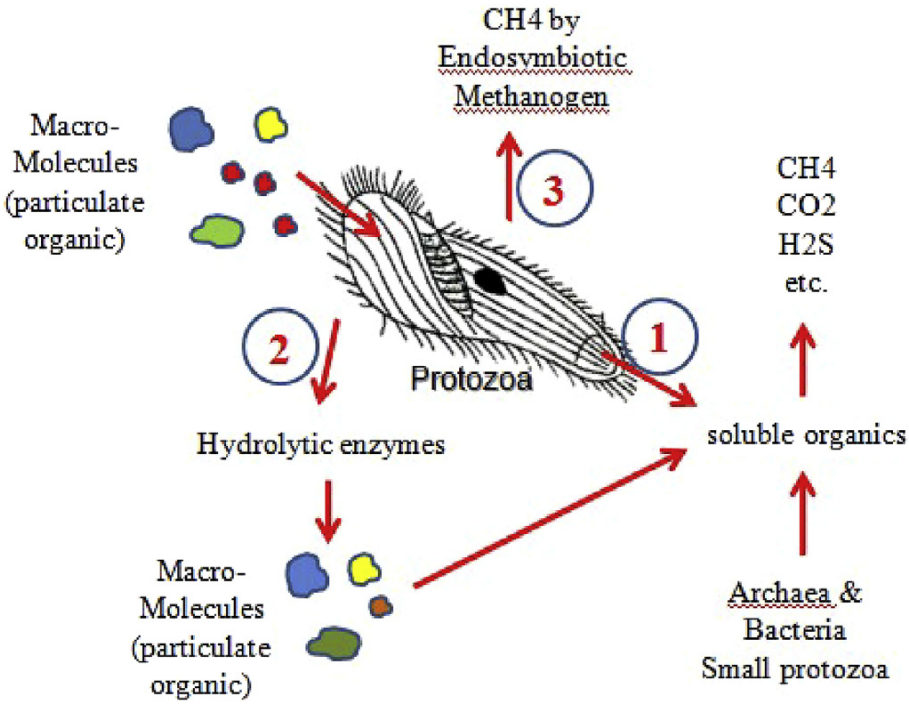

Figure 2-2. A hypothetical scheme role of protozoa in anaerobic digestion of complex organics. (from Prabhakaran et al., 2016)

2.4 Protist community analysis base on 18S rRNA gene sequencing

To investigate ecological role of anaerobic protist, monitoring and understanding of their species and abundance in each ecosystem are needed. Microscopic observation has been used traditionally for analysis of protist community structure and abundance (Curds and Cockburn, 1970; Foissner, 2016). However, identify of anaerobic protist species morphologically by microscopic observation was difficult because some protist species are indiscernibly small (Miyaoka et al., 2017). For this reason, molecular methods based on 18S rRNA genes of eukaryotes instead of microscopic observation have been applying to analyze for protist community structure (Matsunaga et al., 2014; Matsubayasi et al., 2017; Ntougias et al., 2011). In many cases, 18S rRNA gene analysis showed greater diversity of eukaryotes than previously recognized. Indeed, some protist species in river water that overlooked by microscopic observation were detected by 18S rRNA gene sequencing (Liu and Gong, 2012). Matsubayasi et al. (2017) reported clone library of 18S rRNA gene sequencing for eukaryotic community in anaerobic digesters. As result of this analysis, 85% of the sequences clones were less than 97.0% sequence identity to known eukaryotes, indicating that most of the eukaryotes in

anaerobic digesters are largely unknown.

Matsunaga et al. (2014) also investigated microbial eukaryotic community in activated sludge, anoxic/oxic activated sludge, and oxidation ditch, and detected protists that observed microscopically and uncultured eukaryotes by clone library of 18S rRNA gene sequencing. However, some eukaryotes like amoebas that identified by microscopic observations were not found in the clone libraries. This could be resulted from bias of PCR primer and difference of rRNA gene copy number in each eukaryotes cell (Zhu et al., 2005). In particular, difference of rRNA gene copy number is serious concern for 18S rRNA gene sequencing. Therefore, combination of molecular methods and microscopic observation is needed to accurately evaluate for eukaryotic community in wastewater treatment systems (Miyaoka et al., 2017). In addition, the universal eukaryotes specific primer set (e.g., EukA and EukB, Medlin et al., 1988) for 18S rRNA gene sequencing can detect metazoa and fungi also. In case of eukaryotic community analysis including metazoa and fungi, large bias occurs in relative abundance of each group. In general, multicellular organisms such as metazoa and fungi have more rRNA gene copy number than unicellular organism of protists. Thus, 18S rRNA gene sequencing should be separated and analyzed in each eukaryotes group.

Furthermore, in case that 18S rRNA gene sequencing apply for analysis of anaerobic protist community, there are problems derived from using molecular methods, which cannot determine whether the eukaryotes are anaerobic or not, except for known species. This is due to anaerobic protist cannot identify by molecular methods because anaerobic protists don't construct to specific molecular phylogenetic group (Zimorski et al., 2019). Indeed, our previous study could not identify anaerobic eukaryotes by just 18S rRNA gene sequencing because some eukaryotic taxonomic groups include both anaerobic and oxic organisms (Fig. 2-3) (Triadó-Margarit and Casamayor, 2015; Hirakata et al., 2017). Microbial communities in most wastewater treatment systems could be influenced by the immigration of other microorganisms via wastewater. For especially analyze anaerobic protists in wastewater treatment, this problem on immigration must be considered.

2.4.1 18S rRNA gene sequencing using high-throughput sequencing

Recently, high-throughput sequencing of metabarcoding is becoming the standard approach for exploring microbial community structure in various environments. The 16S rRNA amplicon sequencing used to evaluate a wide range of bacterial communities in wastewater treatment processes (Kuroda et al., 2016; Watari et al., 2016). High-throughput sequencing provides a very large number of sequencing reads though only short sequences (Van Dijk et al., 2014). Thus, current methods for high-throughput sequencing of eukaryotic diversity studies rely on sequencing of variable regions of

the 18S rRNA gene. The 18S rRNA genes have nine hypervariable regions (V1 to V9), which can be used for species identification of high-throughput sequencing. The PCR primer sets for high-throughput sequencing that previously reported are described in Table 2-1. Goux et al. (2016) have applied high-throughput sequencing using V1-V5 region of 18S rRNA gene for anaerobic eukaryotic community in farm anaerobic digestion reactor, and reported that relative abundance of the eukaryotic sequences belonging to the phylum Ciliophora (ciliates) showed a positive correlation with the methane content in the reactor headspace. However, several study pointed out that problems on PCR bias of primer set also occurred in High-throughput sequencing (Bradley et al., 2010). Thus, multiple PCR primer sets were used for analysis eukaryotic community structure to examine these PCR and primer bias. In particular, both V4 and V9 regions have been reported to use diversity of eukaryotes found in various environments (Stoeck et al., 2010; Bradley et al., 2010; Dunthorn et al., 2012; Decelle et al., 2014; Inaba et al., 2016).

Table 2-1. 18S rRNA gene specific primer for high-throughput sequencing

primer name		Target region	Sample	Sample condition	Ref
forword	Reverse				
Ek7F	EK516R	V1-V5	Farm anaerobic digestion reactor	anaerobic	Goux et al., 2016
modified Sogin2f	Medinger_rv	V3	Lake water	aerobic	Medinger et al., 2010
P-SSU-316F	GIC758R	V3-V4	Rumen fluid from moose	anaerobic	Ishaq et al., 2014
GIC1080F	GIC1578R	V6-V9			
GIC1184F	GIC1578R	V6-V9			
TAReuk454FWD1	TAReukREV3	V4	Marine anoxic water	anaerobic	Stoeck et al., 2010
V4_1F	TAReukREV3	V4	Faecal samples	anaerobic	Bass et al., 2016
F-566	R-1200	V4-V5	Marine sediment	anaerobic	Hadziavdic et al., 2014
Reuk454FWD1	V4r	V4	Mixed Phototrophic Communities	aerobic	Bradley et al., 2016
V8f	1510R	V9			
1380F	1510R	V9	Marine anoxic water	anaerobic	Stoeck et al., 2009
1389F	1510R	V9			
1391F	EukB	V9	Deep-sea methane seep ecosystem	anaerobic	Pasulka et al., 2016

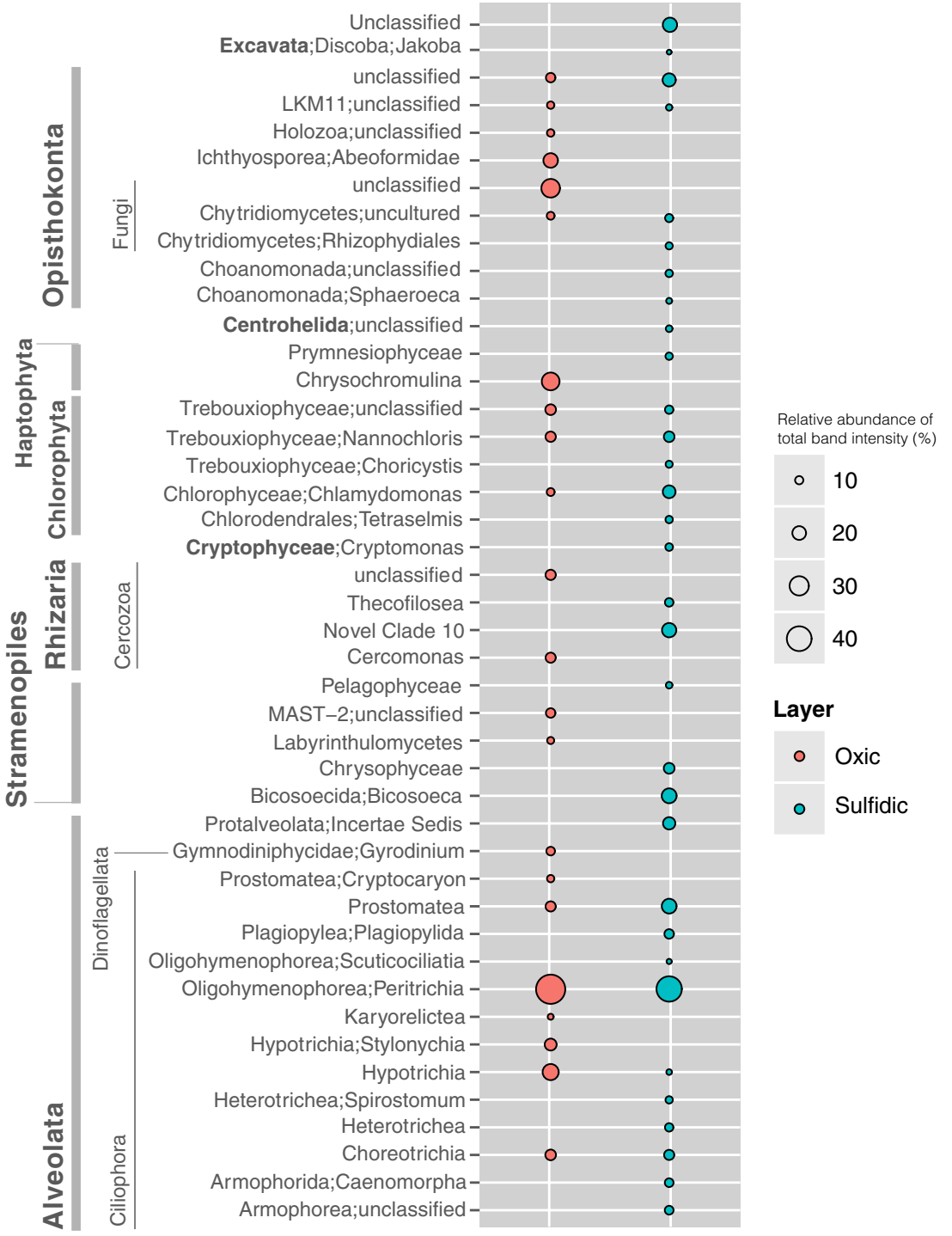

Figure 2-3. Relative abundances of the different taxa identified by DGGE in sulfidic and anoxic (euxinic) stratified karstic lakes and coastal lagoons. (from Triadó-Margarit and Casamayor, 2015)

2.5 Cultivation of anaerobic protist

The isolation and cultivation of anaerobic protist are the major limitations for assessing their roles in anaerobic treatment process. Despite many kinds of anaerobic protists were found in various environments, most of them were still uncultured. Many studies of anaerobic protists were focused on their morphology, phylogeny, and endosymbiotic prokaryotes (Bass et al., 2009; Omar et al., 2016; Lewis et al., 2018). Thus, limited information is available on the cultivation and physiological characteristics of anaerobic protist predation on anoxic ecosystems, except for the parasite species.

Among anaerobic protists, the best-studied species is *T. compressum*. *T. compressum* has been isolated from polluted ditch and sewage treatment plant and cultured monoxenically or axenically using synthetic medium added with living or dead bacteria as substrate (Wagener and Pfennig 1987; Goosen et al. 1990a; Broers et al., 1991; Yamada et al. 1994; Shinzato et al., 2007). *T. compressum* could grow in temperature range of 10-35˚C, in which optimum condition was ranged 25-30˚C (Wagener and Pfennig 1987; Goosen et al. 1990a).

Food selectivity of *T. compressum* was also examined. Schulz (1990) tested 15 chemotrophic and 27 phototrophic bacterial strains as sole food bacteria of protist and concluded that only gram-negative bacteria could support growth of T. compressum. However, Yamada et al. (1994) reported that *T. compressum* could ingest both gram-negative and gram-positive bacteria in addition to archaea. The maximum growth number of *T. compressum* was changed depending on food bactrial species. The highest number of ciliates reached 9300 cells ml^{-1} when *Desulfovibrio vulgaris* was used as food bacteria. These results suggested that growth of *T. compressum* is influenced by nutritions of food bacteria. Their metabolite compositions such as ethanol, acetate, lactate, and formate were also changed depending on food bactrial species (Yamada et al. 1994), and this also indicates that effect of nutrition of food bacteria.

In addition, *T. compressum* are known have sterol requirements as growth factor (Wagener and Pfennig, 1987). The nutritional requirements for sterol and fatty acid as growth factor are known in also other aerobic or parasite protist such as *Paramecium* sp., *Tetrahymena* sp., and *P. shumwayae* (Holz et al. 1962; Skelton et al., 2008). Therefore, understanding not only food bacteria but also nutrition requirement of each protist species is a necessity for establishment of isolation and cultivation of anaerobic protists.

2.6 references

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Chapter III

Temporal variation of eukaryotic community structures in UASB reactor treating domestic sewage as revealed by 18S rRNA gene sequencing

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3.1 Introduction

In order to investigate protists function in anaerobic treatment systems, information about characteristics of protist community structures needs to accumulate at first. Microbial eukaryotes such as protist, fungi, and metazoa play important roles in aerobic wastewater treatment systems. In particular, bacterivorous protists and metazoa contribute to the reduction of sludge production, the improvement of sludge sedimentation, and the quality of effluent water (Madoni, 1994; Pernthaler et al., 2005; dos Santos et al., 2014). Additionally, some fungi, for example phylum Ascomycota, are also known to contribute to denitrification and cellulose degradation (Hayatsu et al., 2008; Baldrian and Valášková, 2008). Eukaryotic communities involved in activated sludge have been widely studied, so their community compositions and populations are used as biological indicators of these processes (Griffiths et al., 2001; Foissner and Berger, 1996).

Eukaryotes have been commonly identified morphologically by microscopic observation. However, identifying protists by microscopic observation is prone to error because some species are fast moving and indiscernibly small (Zhu et al., 2005). Furthermore, most fungi are difficult to identify by microscopic observation. Recently, instead of by microscopic observation, molecular biological techniques such as clone libraries (Miyaoka et al., 2017), quantitative real-time PCR (Bien et al., 2017), fluorescence in situ hybridization (Matsubayashi et al., 2017), and high-throughput sequencing techniques (Simon et al., 2015) have been applied to analyze a greater diversity of eukaryotes in wastewater treatment systems and other enrivonments (e.g. lake and marine). Among them, 18S rRNA gene amplicon sequencing using high-throughput sequencing techniques is reported as an effective and sensitive method for investigating eukaryotic diversity (Tanakaet al., 2014).

High-throughput sequencing provides a very large number of reads though only short sequences are generated (Van Dijk et al., 2014). Thus, current methods for high-throughput sequencing of eukaryotic diversity studies rely on sequencing of variable regions of the 18S rRNA gene. The 18S rRNA genes of eukaryotes have nine hypervariable regions (V1 to V9), which can be used for species identification. A number of recent studies have used different variable regions within the 18S rRNA gene for amplification, including V1-V2 (Mohrbeck et al., 2015), V3 (Medinger et al., 2010), V4, and V9 regions (Stoeck et al., 2010). In particular, both V4 and V9 regions have been used to describe the diversity and variation of eukaryotes found in aerobic wastewater treatment systems (Bradley et al., 2010; Inaba et al., 2016). Previous studies reported that the molecular markers have different characteristics such as V4 region provide more depth and unique reads and V9 region provide wider coverage of higher taxonomic groups.

In contrast to aerobic eukaryotes, limited information is available on the eukaryotic community structures found in anaerobic wastewater treatment systems. Microbial eukaryotes such as protists and fungi also exist and contribute to the degradation of organic matter in anaerobic wastewater treatment systems (Hirakata et al., 2016; McMullan et al., 2001). A few studies have reported that some protists of

phylum Ciliophora positively correlated with the removal of organic matter in anaerobic digesters (Priya et al., 2007; Prabhakaran et al., 2016). Therefore, anaerobic eukaryotic communities, especially protists, are expected to change in response to environmental conditions and may be used as indicators of operational conditions, as with aerobic eukaryotes. However, no reports characterize what eukaryotic species living in anaerobic wastewater treatment system and its temporal variation. Therefore, most species of anaerobic eukaryotes have not been extensively investigated, and the relationships between changing eukaryotic community structures and treatment performance in anaerobic wastewater treatment systems remain unclear. In addition, although 18S rRNA gene amplicon sequencing has been applied to investigate eukaryotic communities in anaerobic environments such as marine (Stoeck et al., 2010) and rumen environments (Ishaq et al., 2014), few studies have used this method for anaerobic wastewater treatment systems (Goux et al., 2016).

Furthermore, there are problems derived from using molecular methods, which cannot determine whether the detected eukaryotes are anaerobic or not, except for known species. Indeed, our previous study could not identify anaerobic eukaryotes by just 18S rRNA gene sequencing because some eukaryotic taxonomic groups include both anaerobic and aerobic organisms (Triadó-Margarit and Casamayor, 2015; Hirakata et al., 2017). In particular, microbial communities in anaerobic wastewater treatment systems may be influenced by the immigration of aerobic microorganisms via wastewater. To characterize the anaerobic eukaryotes involved in wastewater treatment, this issue must be considered.

The primary objective of Chapter III was to investigate eukaryotic community structures in an anaerobic wastewater treatment system. For this purpose, we analyzed eukaryotic communities in an up flow anaerobic sludge blanket (UASB) reactor treating domestic sewage over a two-year operational period. We used 18S rRNA gene amplicon sequencing using two primer pairs, targeting the V4 and V9 regions. Eukaryotic communities in aerobic wastewater treatment systems (i.e., activated sludge) and influent sewage were also analyzed and used as the references for aerobic eukaryotic species to characterize anaerobic eukaryotes. In addition, multivariate statistics were applied to elucidate any correlation between eukaryotic communities and the operational conditions of the UASB reactor using the retrieved anaerobic eukaryotic sequences.

3.2 Materials and methods

3.2.1 Sample collection

Sludge samples of 50 mL were collected over two years (October 2010–October 2012, Fig. 3-1a) from a sampling port 1.278 m above the bottom of the UASB reactor. The reactor had a total volume of 1,178 L, was 4.7 m in height, and was located at a domestic sewage treatment center of Nagaoka City, Japan. The UASB reactor was operated without temperature control. To activate the microorganisms responsible for sulfur redox cycles, the system was fed with raw sewage that was supplemented with

50–150 mg-S L^{-1} sodium sulfate. Additional details on the UASB reactor have been previously described (Tandukar et al., 2007). To classify the anaerobic and aerobic eukaryotes species, activated sludge and influent sewage were collected from the same domestic sewage treatment center in February 2017. The collected samples were concentrated by centrifugation at 12,000 rpm and removed supernatant, then immediately stored at −20 °C for 4-6 years until DNA extraction was performed.

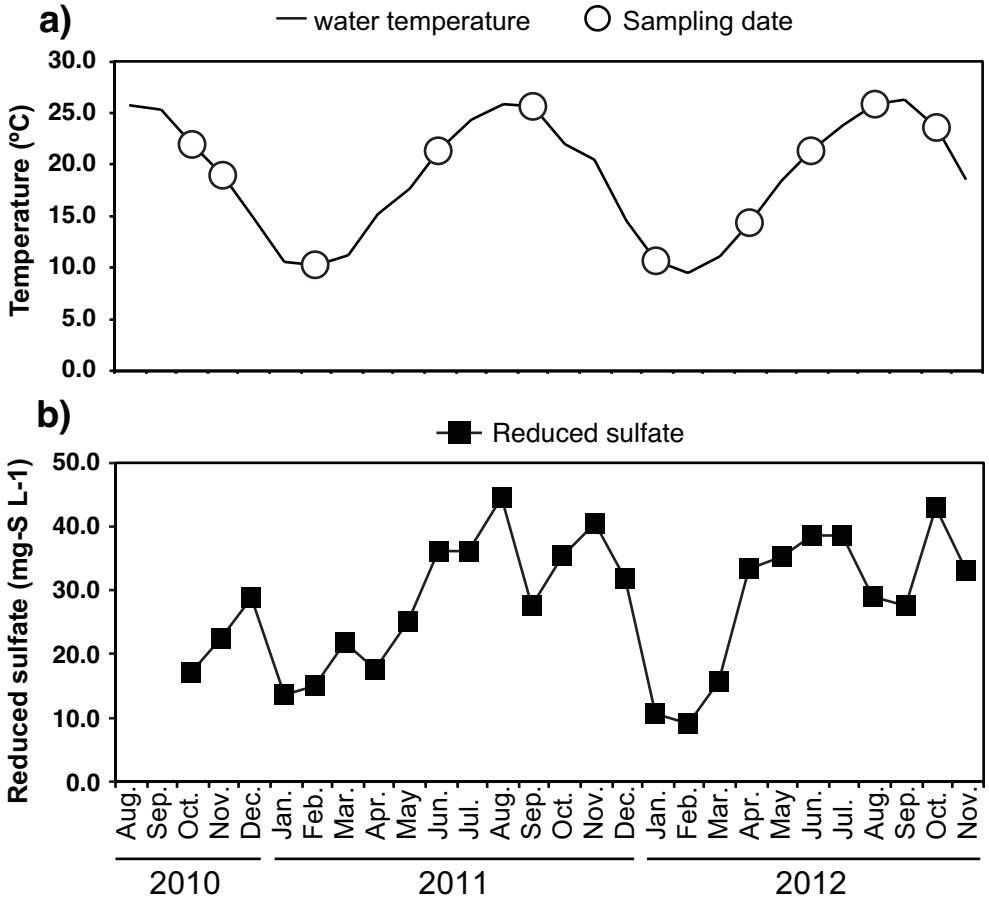

Figure 3-1. Time courses of (a) water temperature and (b) reduced sulfate (sulfide) of the UASB reactor and sampling date of sludge samples.

3.2.2 Measurement of environmental parameters

The water temperature and the pH were measured using a pH meter (HM-20P; TOA DKK, Tokyo, Japan). The oxidation-reduction potential (ORP) were measured using an ORP meter (RM-20P; TOA DKK). The chemical oxygen demand (COD) concentration was determined using a HACH water quality analyzer (DR2500; HACH, Loveland, CO, USA). The suspended solid (SS) concentration was also measured using a glass fiber filter (0.4 μm, GB140; Advantec, Tokyo, Japan). The sulfate concentrations were determined by a high-performance liquid chromatography (HPLC) system (LC

20-ADsp; Shimadzu, Kyoto, Japan). The sulfide concentration was measured according to the standard methods published by the Japan Sewage Works Association (Japan Sewage Works Association, 1997).

3.2.3 DNA extraction, PCR amplification, and 18S rRNA gene sequencing

Genomic DNA was extracted from the collected samples using a FastDNA SPIN Kit for Soil (MP Biomedicals, Carlsbad, CA, USA), according to the manufacturer's protocol. The DNA concentration was determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Amplifications of V4 and V9 regions of 18S rRNA genes were performed using eukaryote-specific primer pairs of V4_1F (5'-CCAGCASCYGCGGTAATWCC-3') - TAReukREV3 (5'-ACTTTCGTTCTTGATYRA-3') and Euk1391F (5'-GTACACACCGCCCGTC-3') - EukBR (5'-TGATCCTTCTGCAGGTTCACCTAC-3'), respectively (Stoeck et al., 2010; Bass et al., 2016). The adapters for Illumina MiSeq sequencing were attached for each primer according to previous study (Caporaso et al., 2012). Premix Ex Taq Hot Start Version (TaKaRa Bio Inc., Shiga, Japan) was used for PCR amplification. The following were the conditions of PCR amplification. For amplification of V4 region, 5 min at 94 °C; 15 cycles of 30 s at 94 °C, 45 s at 53 °C, and 1 min at 72 °C; 20 cycles of 30 s at 94 °C, 45 s at 48 °C, and 1 min at 72 °C; with a final extension step of 10 min at 72 °C. For amplification of V9 region, 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 57 °C, and 1 min at 72 °C; with a final extension step of 10 min at 72 °C. The amplicon was purified using an Agencourt AMPure XP Kit (Beckman Coulter, Brea, CA, USA) and concentrations were measured using a BioAnalyzer DNA 1000 (Agilent Technologies, Santa Clara, CA, USA). 18S rRNA gene sequencing was conducted using a MiSeq Reagent Kit v2 nano and a MiSeq system (Illumina, San Diego, CA, USA).

3.2.4 Data Analysis

Sequence reads were processed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.0 (Caporaso et al., 2012). Sequence reads with low quality scores (Phred quality score ≤ 30) were eliminated using the Trimmomatic v0.33 program; specifying a sliding window of 4 with average Phred quality of 30 and 60 as the minimum read length to be conserved for quality control (Bolger et al., 2014). Paired-end sequence reads were then assembled using the paired-end assembler within the Illumina sequence software package (PANDAseq), and at least 20 bp overlapping region was retained (Masella et al., 2012). Putative chimeric sequences were detected and removed using UCHIME software (Edgar et al., 2011). Operational taxonomic units (OTUs) clustering at 97% sequence identity was conducted with the de novo strategy using the UCLUST algorithm (Edgar et al., 2010). Taxonomic classifications were determined using the SILVA database 128 (Quast et al., 2013) and BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in the National Center for Biotechnology Information (NCBI) database. The OTU that assigned to prokaryotes using SILVA and NCBI database were excluded from

multivariate statistics. Alpha diversity index of eukaryotic sequences from each sample was calculated at a subsampling depth of lowest reads from each amplicon library, protist and fungi sequences. The phylogenic tree was constructed in MEGA software using neighbor-joining methods (Tamura et al., 2013).

After detrended correspondence analysis (DCA) was performed to determine the appropriate type of model for direct gradient analysis, canonical correspondence analysis (CCA) or redundancy analysis (RDA) were performed to investigate correlations between eukaryotic communities and environmental factors using the 'vegan' R package (Oksanen et al., 2013). In this study, CCA analysis was used because gradient length was calculated as 3.1. The value is greater than 2, it is suitable for using CCA (Ter Braak and Verdonschot, 1995). A Monte Carlo test was used to check the significance of multivariate analysis using the 'ade4' R package (Dray et al., 2007). These analyses included the environmental parameters of the UASB reactor, and 17 anaerobic protist genera, representing at least 0.3% mean relative abundance per sample. The change of protist and fungi community structure in the UASB reactor was evaluated by principal coordinates analysis (PCoA) based on Bray-Curtis distance. The difference of individual eukaryotes groups at a different time was determined by one-way analysis of variance (ANOVA) or Welch's t-tests.

3.2.5 Nucleotide sequence accession numbers

Sequence data were deposited in the DDBJ nucleotide sequence database under accession numbers DRA007151.

3.3 Results

3.3.1 Overall eukaryotic communities determined by V4 and V9 regions of 18S rRNA gene sequencing

In this study, eukaryotic community structures were analyzed for 10 samples from a UASB reactor (Fig. 3-1a), influent sewage, and activated sludge, based on 18S rRNA gene sequencing. A total of 180,678 sequences of the V4 region amplicon library and 340,054 sequences of the V9 region amplicon library were obtained (Supplementary Table S3-1). The phylogenetic affiliations of all sequences in each ecosystem were classified as archaea, bacteria, protist, fungi, metazoa, and algae at the kingdom or domain level (Fig. 3-2). The results using the V4 region-specific primer pair showed that the dominant group was fungi in the UASB reactor and influent sewage, whereas protists were dominant in activated sludge. The relative abundances of fungi were 76.1% and 50.8% of the total number of sequences in the UASB reactor and influent sewage, respectively. The relative abundance of fungi in the UASB was continuously high throughout the year (Supplementary Fig. S3-1a). In contrast, the relative protist abundance was very low in the UASB reactor, accounting for 3.8% of all sequences. Additionally, the relative abundance of algae and metazoa were detected as 0.3–3.9% and 7.1–9.8%, respectively.

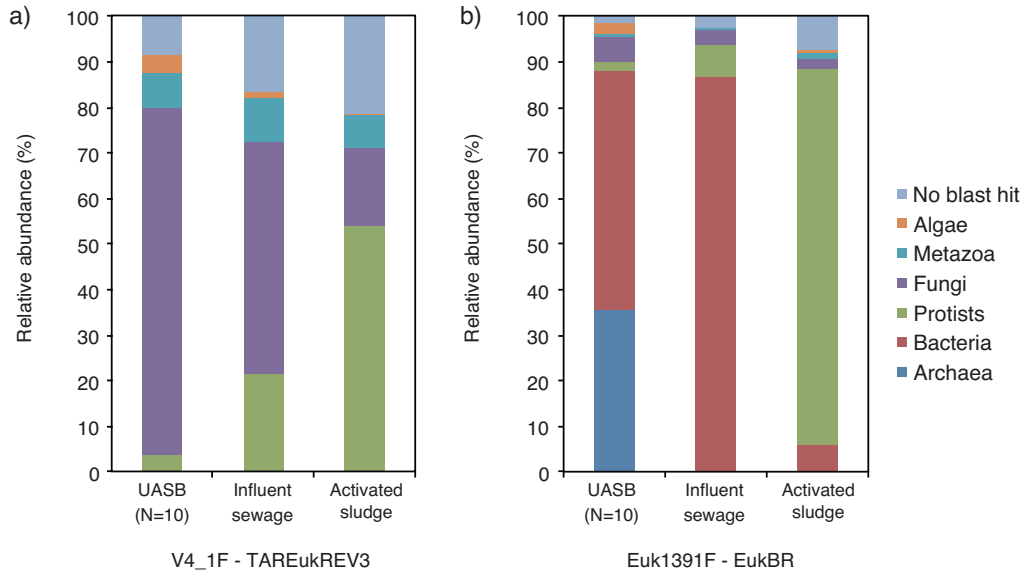

Figure 3-2. Relative abundance of (a) V4 and (b) V9 region amplicons assigned to kingdom or domain level in the UASB reactor (N = 10), influent sewage, and activated sludge. Sequence reads that are not classified into any known group were labeled as "No blast hit."

However, the results using the V9 region-specific primer pair showed that not only eukaryotic but also a large portion of prokaryotic sequences also detected from the UASB reactor and influent sewage (Fig. 3-2, Supplementary Tables S3-2, S3-3). The bacteria and archaea in the UASB reactor were accounted for 52.2% and 35.6% of total number of sequence, respectively. On the other hand, protists were dominant in activated sludge samples, where the relative abundance of bacteria was low.

To evaluate eukaryotic communities, sequences classified as archaea and bacteria were excluded from the rest of the analyses. Thus, in the present study, a total of 169,385 and 45,510 eukaryotic sequence reads were generated from the V4 and V9 amplicon libraries, respectively. The alpha diversity was calculated using the lowest sample sizes of eukaryotic sequences for comparison of each amplicon library (Supplementary Table S3-2). The values of species richness estimates, observed species, Chao1, and ACE were higher in the V4 amplicon library than in the V9 amplicon library. In both amplicon libraries, these values were greater in the UASB reactor than in activated sludge.

3.3.2 Protist Community Structures

Taxonomic classification of the protist community structures analyzed by V4 and V9 region-specific primer pairs were compared at the phylum level (Fig. 3-3). In the present study, a total of 3,204 OTUs and 691 OTUs of V4 and V9 amplicon libraries, respectively, were identified (Supplementary Table S3-4). In the V4 amplicon library, the dominant heterotrophic protist groups in the UASB reactor were phyla Ciliophora and Amoebozoa, with average relative abundances of 27.2% and 10.6%, respectively. Phyla Apicomplexa, Ichthyosporea, and

Perkinsozoa, which are known as parasitic protists (Ajonina et al., 2012; Mangot et al., 2011; Mendoza et al., 2002), were also detected in the UASB reactor at average relative abundances of 18.6%, 11.0%, and 10.6%, respectively. The heterotrophic protists identified included phyla Cercozoa, Sulcozoa, Bicosoecida, Choanomonada, Dinoflagellata, and Metamonada (>1% on average).

In the V9 amplicon library from the UASB reactor, phyla Metamonada, Apicomplexa, Amoebozoa, and Ciliophora were dominant, displaying average relative abundances of 23.9%, 21.8%, 12.5%, and 11.0%, respectively. Phyla Ciliophora, Ichthyosporea, Perkinsozoa, and Sulcozoa were found at lower levels in the V9 amplicon library than in the V4 amplicon library. In contrast, phyla Metamonada, Heterolobosea, and Euglenozoa were more abundant in the V9 amplicon library. Although changes in community composition at phylum level were observed in the UASB reactor over the two years, the effect of seasonal changes on protist communities (e.g., winter and summer) was unclear in both amplicon libraries. The alpha diversity indexes of protist sequences includes observed species, Chao1, and ACE had also changed regardless of seasonality in the UASB reactor throughout the two years, while simpson and shannon indexes were not significantly different (Supplementary Table S3-4). This suggested that protist community could be affected by operational condition than seasonality.

In the V4 amplicon library from influent sewage, the dominant groups were phyla Ciliophora and Cercozoa and the RT5iin25 group, with relative abundances of 54.5%, 9.9%, and 20.5%, respectively. Phyla Ciliophora and Euglenozoa were dominant in the V9 amplicon library from influent sewage, with relative abundances of 41.9% and 37.2%, respectively. In the V9 amplicon library from the UASB reactor and influent sewage, Excavata groups were detected as being more dominant than in the V4 amplicon library. The major groups in activated sludge were similar in both V4 and V9 amplicon libraries, where phyla Ciliophora, Cercozoa, and

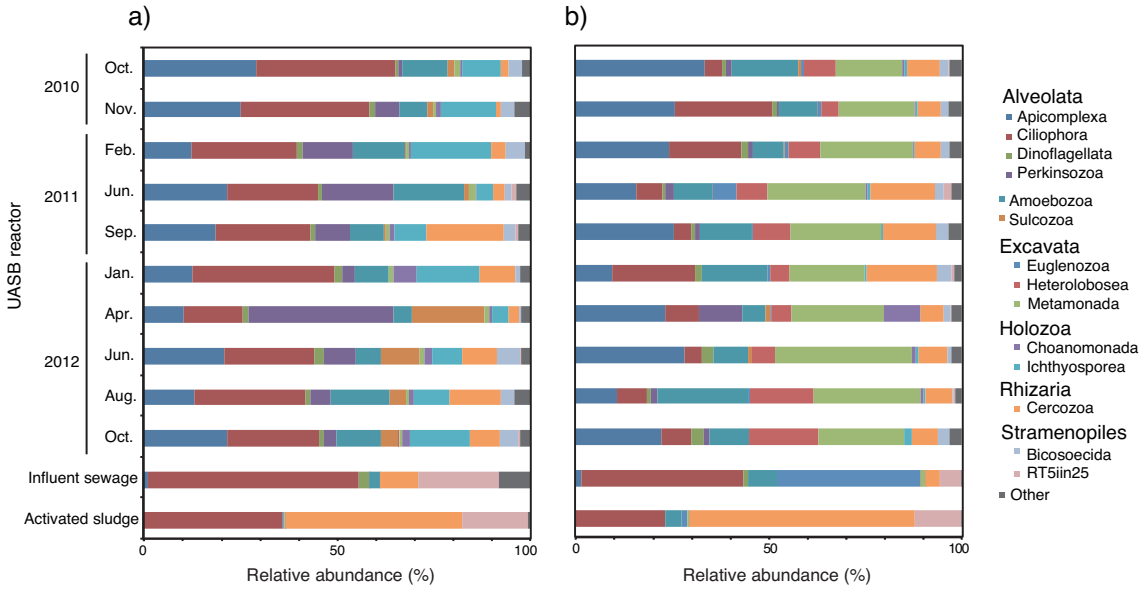

Figure 3-3. Relative abundance of (a) V4 and (b) V9 region amplicons assigned to protist phylum level in all samples from the UASB reactor, influent sewage, and activated sludge.

RT5iin25 groups were dominant.

3.3.3 Temporal variation in anaerobic protist communities

To determine the factors that influence temporal changes in protist communities in UASB reactors, multivariate statistical analysis was conducted to assess correlation between the major anaerobic protists and environmental parameters. For the analysis, the V4 amplicon libraries were used due to the primer pair's specificity for eukaryotic sequences. Anaerobic protist genera in the UASB reactor were identified by using eukaryotic sequence obtained from activated sludge and influent sewage as reference of aerobic protist (Supplementary Fig. S3-2). In the UASB reactor, some protists were from known aerobic genera such as *Epistylis*, *Telotrochidium*, *Tetrahymena*, *Vorticella,* within phylum Ciliophora (Foissner, 2016); *Phalansterium* and *Saccamoeba* within phylum Amoebozoa (Cavalier-Smith et al., 2004); *Cercomonas*, *Heteromita*, and *Rhogostoma* within phylum Cercozoa (Howe et al., 2011; Ekelund, 2002); and *Protoperidinium* within phylum Dinoflagellata (Yamaguchi and Horiguchi, 2008) which were frequently detected. These protist genera were also detected in influent sewage and activated sewage. The parasitic protist *Cryptosporidium* (phylum Apicomplexa) was detected in both influent sewage and in the UASB reactor. Although genera *Acanthamoeba* and *Tracheloraphis* that could prey other protist cell (Anderson et al., 2005; Hamels et al., 2005) were also detected in the UASB reactor, these protist and other protist genera were not correlated. These common protist genera in both influent sewage and the UASB reactor accounted for 25.8% of the total protist sequences from the UASB reactor. In contrast, the general anaerobic protists of *Metopus* (phylum Ciliophora) and *Trimastix* (phylum Metamonada) (Fenchel and Finlay, 1990; Hampl et al., 2008) were detected only in the UASB reactor. Additionally, protist genera belonging to phyla Sulcozoa, Bicosoecida, Choanozoa, and Metamonada were exclusively found in the UASB reactor.

The correlations between anaerobic protist genera and treatment performance in the UASB reactor were examined using CCA (Fig. 3-4). CCA includes the anaerobic protist genera that were specific to the UASB reactor (Supplementary Fig. S3-2) and the environmental parameters (Supplementary Table S3-5). As shown by CCA, genus *Subulatomonas* (phylum Sulcozoa) positively correlated with effluent in COD, SS, and sulfide, whereas *Platyophrya* and *Cyclidium* (phylum Ciliophora) showed negative correlations. Furthermore, comparisons of variation of operational condition and alpha diversity over two years showed that value of estimated species, Chao1 and ACE of protist in V4 amplicon library seemed high when COD concentration of UASB effluent was low (Supplementary Table S3-4, S3-5). These results also supported that some protist population changed in response to environmental conditions. There were no protist genera that were correlated clearly with water temperature. The one-way analysis of variance (ANOVA) was also performed to analyze difference of relative abundance of protist genera at different season; summer (23.6 - 26.4˚C); winter (10.3 - 14.5˚C); spring and fall (18.7 - 21.6˚C). However, no significant differences were found (data not shown). The principal coordinate analysis (PCoA) showed that protist community structures were not influenced by temperature and reduced sulfate (Supplementary Fig. S3-3).

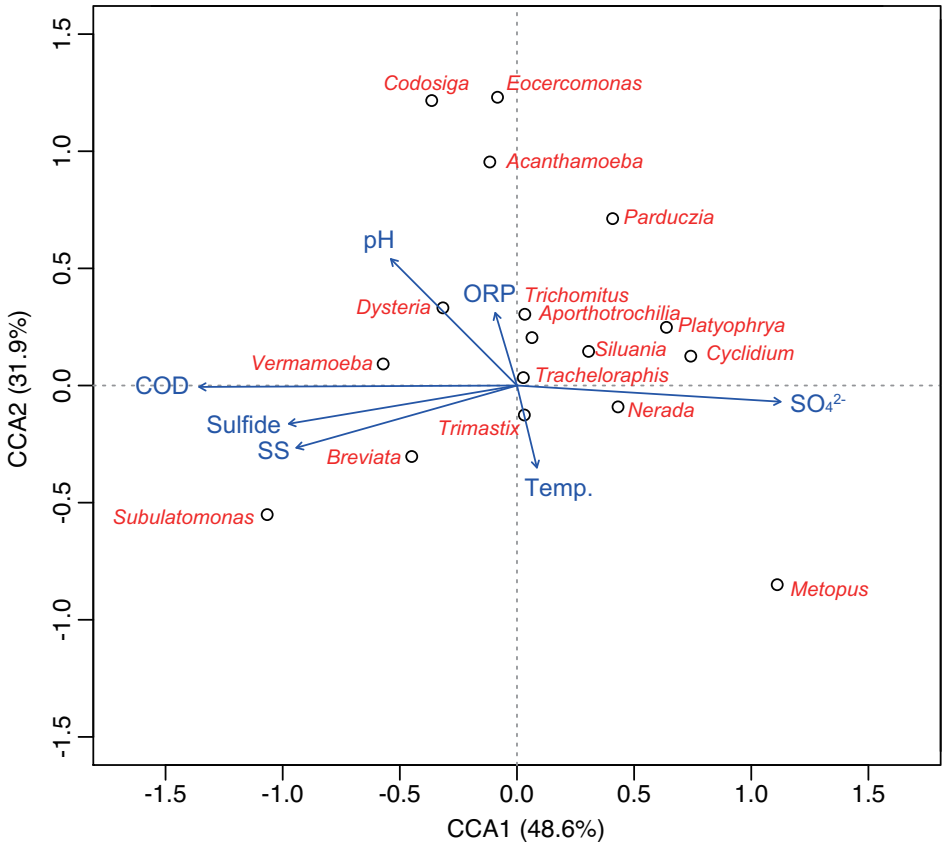

Figure 3-4. Canonical correspondence analysis based on treatment performance and on the 17 unique protist genera of the UASB reactor having a mean relative abundance per sample of 0.3% or above.

3.3.4 Fungal community structures

Taxonomic classification of the fungal community structures analyzed by V4 and V9 region-specific primer pairs were compared at the phylum level (Fig. 3-5). In total, 15,109 and 527 OTUs were identified from fungal amplicons using V4 and V9, respectively (Supplementary Table S3-6). These sequence reads were classified as phyla Ascomycota, Basidiomycota, Chytridiomycota, Discicristoidea, Hyphochytriomycetes, and uncultured LKM11 and LKM15 groups in phylum Cryptomycota. The relative abundance of the fungi was 17.4–85.8% of the V4 amplicon library in all sequences of each sample. In the V4 amplicon library from the UASB reactor, the dominant fungi were the LKM11 and LKM15 groups, with average relative abundances of 38.8% and 31.7% of all fungal sequences, respectively. In the activated sludge, the LKM11 group was most abundant, accounting for 81.5%. Phylum Ascomycota and the LKM11 group were also detected in the influent sewage, with relative abundances of 60.5% and 34.2%, respectively. In comparison with the V4 amplicon library, the V9 amplicon library showed a drastically different composition (Fig. 3-5), and phylum Ascomycota was dominantly detected in all samples. Within sequences belonging to phylum

Ascomycota in the V9 amplicon library from the UASB reactor, genus *Candida* accounted for 84.7%. The community structures and alpha diversity of fungi had changed regardless of seasonality and reduced sulfate in the UASB reactor throughout the two years as with protist community (Supplementary Table S3-6). The PCoA results supported that fungi community structures were not influenced by temperature and reduced sulfate (Supplementary Fig. S3-4).

Phylogenetic analyses of predominant OTUs belonging to uncultured LKM11 and LKM15 groups in phylum Cryptomycota were performed using 18S rRNA gene sequences from the V4 amplicon library (Supplementary Fig. S3-5). The abundance of OTUs belonging to the LKM11 group varies between samples. The OTU denovo13315 was detected only in the UASB reactor, with an average relative abundance of 8.1%. The OTU denovo4805 was most dominant in activated sludge, at a relative abundance of 49.5%, and was hardly detected in other samples. These results suggested that these two species, OTU denovo13315 and denovo4805, could live in the UASB reactor or activated sludge. Additionally, the OTU denovo9985 was dominant in influent sewage. Other OTUs denovo15978, 18515, 20531, and 22061 belonged to the LKM11 group and were detected in all samples. By contrast, OTUs belonging to the LKM15 group were detected only in the UASB reactors. The OTU denovo23550 was the most dominant, accounting for 97.6% of all OTUs belonging to the LKM15 group.

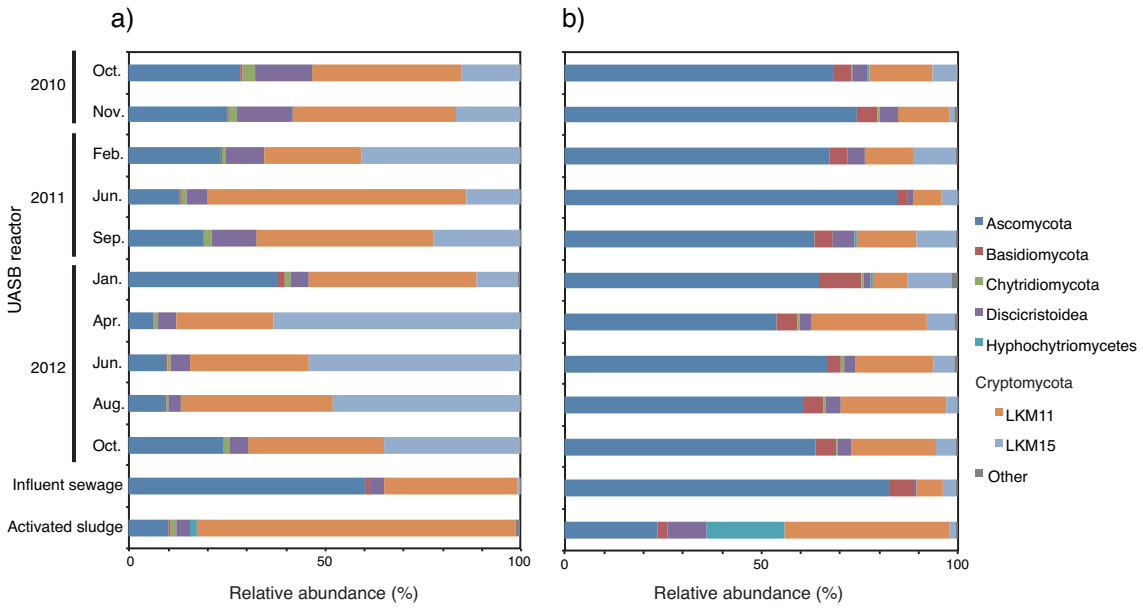

Figure 3-5. Relative abundance of (a) V4 and (b) V9 region amplicons assigned to fungi phylum level in all samples of UASB reactor, influent sewage, and activated sludge.

3.4 Discussion

In this study, the eukaryotic community structures in a UASB reactor fed with domestic sewage were investigated by amplicon sequencing of the V4 and V9 regions of 18S rRNA gene. Eukaryotic communities

involved in anaerobic wastewater treatment systems have remained poorly understood in comparison to aerobic processes. To the best of our knowledge, this is the first study of eukaryotic communities involved in anaerobic wastewater treatment that were characterized by comparison between the UASB reactor and influent sewage based on 18S rRNA gene sequencing analysis. Additionally, this study evaluated the applicability of V4 and V9 region-specific primer pairs to characterize eukaryotic communities in the UASB reactor via high-throughput sequencing. The V4 and V9 region-specific primer pairs have been used recently to describe the diversity and communities of eukaryotes in several studies as their flanking regions are well conserved (Massana et al., 2014; De Vargas et al., 2015). The observed species, Chao1, and ACE indexed of eukaryotic communities were higher in the V4 amplicon library than V9 amplicon library (Supplementary Table S3-2). This could have resulted from the V4 region amplicon being longer than the V9 region amplicon (V4 : 341 bp and V9 : 102 bp). In addition, the V4 region-specific primer pair of V4_1F and TAReukREV3 could be used to specifically amplify eukaryotic sequences from all samples, making them available for investigations of eukaryotes in anaerobic treatment systems.

The V9 region-specific primer pair of Euk1391F and EukBR amplified eukaryotic and prokaryotic sequences from samples collected from the UASB reactor and influent sewage (Fig. 3-2, Supplementary Table S3-2). Although there is possibility that detection of prokaryotes resulted from the combined effects of read errors during PCR and sequencing, PCR chimera formation, a total of 288,723 sequences assigned to prokaryotes passed QC (Supplementary Table S3-1). The V9 region specific primer amplified relatively short sequences (<500 bp) in comparison to Sanger sequencing based methods like clone library (>1 kb). In theoretically, short read sequences minimize the occurrence of chimera formation (Stoeck et al., 2010). Additionally, contig length of eukaryotes and prokaryotes obtained from V9 amplicon libraries were different for 123 ± 13 bp and 103 ± 17 bp, respectively. Thus, detection of large number of prokaryotic sequences is considered not for PCR chimera formation and read errors. The forward primer of Euk1391F is targeted at highly conserved rRNA gene sequence regions among the three domains, meaning that non-eukaryotic sequences have often been amplified (Stoeck et al., 2010). The dominant prokaryotes detected by the primer pair of Euk1391F and EukBR from the UASB reactor and influent sewage were genera *Pseudomonas*, *Syntrophobacter*, and *Arcobacter* within phylum Proteobacteria, and *Methanomassiliicoccus* and *Methanobacterium* affiliated with phylum Euryarchaeota. These anaerobes and facultative anaerobes that have 0-1 and 2-4 mismatches with Euk1391F and EukBR, respectively (Supplementary Table S3-3, Fig. S3-6). Despite previous studies using the same V9 region-specific primer pair for both anoxic and aerobic samples, the relative abundance of prokaryotes was very low in all sequences (<1%) (Stoeck et al., 2009; 2010). The high detection ratio of prokaryotes in the present study may be due to the microbial community including a high proportion of prokaryotes that have no, or low levels of, mismatches with Euk1391F and EukBR. Those prokaryotes were detected from the UASB reactor samples based on 16S rRNA gene sequencing (Watari et al.,

2017; Kuroda et al., 2015). Therefore V9 region-specific primer pair of Euk1391F and EukBR was not suitable for analysis of eukaryotic communities in the UASB reactor.

Protists and fungi are known to be the dominant eukaryotes in anaerobic environments (Hackstein et al., 1999; Müller, 1993). Nevertheless, not only protists and fungi but also metazoa and algae were detected in the UASB reactor (Fig. 3-2). In V4 and/or V9 amplicon libraries, phyla Charophyta and Chlorophyta in algae and phyla Nematoda and Arthropoda in metazoa were the dominant groups in the UASB reactor and were also detected in influent sewage (Supplementary Figs. S3-7 and S3-8). The members of phyla Chlorophyta and Charophyta are known as either photosynthetic or aerobic heterotrophic organisms (Chiu et al., 2015; Khataee et al., 2010). Some species of phylum Nematoda were often observed in wastewater treatment plants and raw municipal wastewater, at the egg stage of their life cycle (Ayed et al., 2009). However, phylum Arthropoda that known to be intolerant of anoxic conditions and was therefore probably introduced into the UASB reactor via influent sewage, in which they were also detected. Furthermore, aerobic, parasitic protists and some fungal species were detected in the UASB reactor and influent sewage (Fig. S3-3, 3-5; Supplementary Figs S3-2, S3-5); this indicates that the presence of these species in influent sewage affects eukaryotic communities in the UASB reactor. These data suggest that eukaryotic species in influent sewage should be considered during identification of anaerobic eukaryotes.

The result of this study showed that 18S rRNA gene amplicon sequencing could reveal larger numbers of protist species in the UASB reactor and activated sludge than the microscopic observations (Hirakata et al., 2016; Foissner, 2016) and clone libraries used in previous studies (Matsunaga et al., 2014; Prabhakaran et al., 2016). Although many protist groups that are barely observable microscopically were detected by 18S rRNA gene amplicon sequencing, V4 and V9 amplicon libraries detected different compositions within protist sequences. Consistent with our previous study, phylum Ciliophora, which was dominantly observed microscopically in the UASB reactor (Hirakata et al., 2016), was the most dominant group in V4 amplicon library. However, phylum Metamonada, which was not found in microscopic observations, were more dominant than phylum Ciliophora in the V9 amplicon library from the UASB reactor. In addition, some protist groups that were detected at low levels in the V4 amplicon library were strongly detected in the V9 amplicon library (e.g., phylum Metamonada, Heterolobosea, and Euglenozoa). Previous studies have reported that V4 and V9 region-specific primer sequences preferentially detected different protist groups (Stoeck et al., 2010; Tragin et al., 2018). This may be caused by the different detection biases of each primer pair. These protist groups, especially phylum Metamonada, were the most likely to be overlooked microscopically; therefore, their populations in UASB reactors should be examined in future studies.

The protist community structures in the UASB reactor were distinctly different and composed of a wide range of taxonomic groups, compared with influent sewage and activated sludge (Fig. 3-3). Phyla Cercozoa, Amoebozoa, and Ciliophora were detected in both samples. These phyla are found frequently in both aerobic and anaerobic environments (Bernard et al., 2000; Cavalier-Smith et al., 2004; Triadó-Margarit and Casamayor,

2015). In addition, protist groups including these phyla detected from influent sewage and activated sludge in this study were also detected in previous studies throughout the year (Matsunaga et al., 2014; Zahedi et al., 2019). This result showed that anaerobic protist species could be retrieved by using these eukaryotic sequences obtained from activated sludge and influent sewage as a reference of aerobic species (Supplementary Fig. S3-2). Contrary to these, the protist phyla Sulcozoa, Bicosoecida, Choanozoa, and Metamonada were found exclusively in the UASB reactor, and not in the influent sewage. These protists were previously found in many anaerobic environments such as animal gut (Mostegl et al., 2012), anoxic sediment of saline lake (Takishita et al., 2007), anoxic zone of freshwater lake (Lepère et al., 2016), and marine environments (Walker et al., 2006; Wylezich et al., 2012; Yubuki et al., 2015). Thus, these protists are anaerobic and could live in the UASB reactor.

The parasitic protists, such as phyla Ichthyosporea and Perkinsozoa, were detected only in the UASB reactor in both amplicon libraries. Species of phyla Ichthyosporea and Perkinsozoa were previously detected in marine (Takishita et al., 2005) and freshwater environments (Matsubayashi et al., 2017) and have free-living stages and cyst stages during their life cycles (Mendoza et al., 2002; Mangot et al., 2011). Although it is unclear whether those organisms occur in free-living or parasitic forms in the UASB reactor, this result suggested that these species could grow in the UASB reactor.

Some correlation was found between certain anaerobic protist genera and the environmental parameters of the UASB reactor (Fig. 3-4). The genera *Cyclidium* and *Platyophrya* (phylum Ciliophora) were negatively correlated with COD and SS concentrations of effluent, suggesting the importance of these protists as indicators of good treatment performance in UASB reactors. These protist genera are bacterivorous species in anaerobic environments (Clarke et al., 1993; Petz et al., 2007), and may contribute to the degradation of particulate organic matter. Notably, a positive correlation between genus *Cyclidium* and COD removal, volatile fatty acid (VFA) concentration, and gas production, have been observed in anaerobic digesters previously (Priya et al., 2007; Prabhakaran et al., 2016). In contrast, genus *Subulatomonas* (phylum Sulcozoa) and effluent COD and SS were positively correlated. The genus *Subulatomonas,* isolated from anoxic marine sediment, can grow anaerobically with mixed bacteria as a substrate (Katz et al., 2011). Additionally, Xie et al (2018) investigated microbial communities in coastal sediment impacted by oil pollution, reporting that the *Subulatomonas* genus was dominant in sediments containing high concentrations of oil. Therefore, it is possible that these species preferentially grow under high organic matter concentrations and might be considered as indicators of poor treatment performance in the UASB reactors.

The seasonality of most protist genera in the UASB reactor was unclear. However, growth efficiency of protists was influenced by water temperature (Straile, 1997), while some anaerobic protist species showed increased growth rates at temperatures higher than 20 ˚C (Wagener and Pfennig, 1987). Thus, the total protist population of the UASB reactor may be different in every season, resulting from water temperature changes. To

use protists as biological indicators of the UASB reactor, further studies are required to establish associations between protist diversity, population changes, and environmental parameters in detail.

The effect of reduced sulfate on the eukaryotic community was also not found in the UASB reactor even though sulfide could be an important factor of growth inhibition of microorganism. The reduced sulfate increased during high temperature period, and decreased during low temperature period (Fig. 3-1). No significant differences of relative abundance of protist genera were found between low reduced sulfate period (10.6 - 21.8 mg-S L^{-1}) and high reduced sulfate period (28.9 - 42.9 mg-S L^{-1}) (data not shown). The inside of the UASB reactor is sulfide-rich environment compared than natural environment such as freshwater lake because sulfide-rich sewage was fed into the UASB reactors for a long time and sulfate reduction was always occurred (Tandukar et al., 2007; Aida et al., 2015; Hirakata et al., 2016). This situation possibly resulted in the selective construction of the sulfide-resistant protist community, thus they were not susceptible to sulfide. This phenomenon could also result in the fungi community in the UASB reactor.

Fungi were the dominant eukaryotes in the UASB reactor (Fig. 3-2). The dominant fungal groups were different in the V4 and V9 amplicon libraries, likely due to differences in the detection biases of the primer pairs, as discussed above. In the V9 amplicon library from the UASB reactor, the dominant fungus group was genus *Candida* (phyla Ascomycota), which was found in the anaerobic digester and known to grow under anaerobic conditions (Matsubayashi et al., 2017). On the other hand, the uncultured fungal groups of LKM11 and LKM15 in phylum Cryptomycota were dominantly detected in the V4 amplicon library. Although the uncultured LKM11 group in phylum Cryptomycota was detected in all samples, the dominant OTUs of each environment were different (Supplementary Fig. S3-5). This could have resulted from the LKM11 group including both aerobic and anaerobic species. The LKM11 group was previously detected in activated sludge treating domestic sewage (Matsunaga et al., 2014), anaerobic digester (Matsubayashi et al., 2017), anoxic sediments (Dawson et al., 2002), and freshwater lake (Lepère et al., 2010). Some members of the LKM11 group in freshwater are expected to be parasitic fungi or be involved in the decomposition of detritus (Lepère et al., 2007; Simon et al., 2015). In contrast, the LKM15 group was detected only in the UASB reactor, indicating their ability to survive and grow in this environment. Sequences of the LKM15 group were found in anoxic environments such as lake or pond sediments (Wurzbacher et al., 2016). However, the functions of the LKM11 and LKM15 groups in the sewage treatment process are still largely unknown. Our results showed that some members of the LKM11 and LKM15 groups were independent in the UASB reactor from the influent sewage and may be involved in organic degradation in anaerobic wastewater treatment systems. Their functions should be examined in more detail in future studies.

3.5 Summary of this Chapter

In summary, this study revealed protist and fungi communities existing in a UASB reactor treating domestic sewage, using the V4 and V9 regions of 18S rRNA for gene amplicon sequencing. The V4

region-specific primer pair was suitable for analysis of eukaryotic communities in the UASB reactor because eukaryotic sequences were specifically amplified. Eukaryotic community structures in the UASB reactor were influenced by the immigration of eukaryotes via influent sewage, but were clearly different from influent sewage and activated sludge. The changes of protist and fungi community structure in the UASB reactor were not influenced by seasonality. The most dominant protist groups in the UASB reactor were phylum Ciliophora throughout the two years. Multivariate statistics indicated that protist genera *Cyclidium*, *Platyophrya* (phylum Ciliophora) and *Subulatomonas* (phylum Sulcozoa) correlated with chemical oxygen demand and suspended solid concentration, and could be used as bioindicators of treatment performance. In addition, uncultured eukaryotes such as parasitic protists and LKM11 and LKM15 groups of fungi were exclusively detected in the UASB reactor. The physiological roles of these uncultured eukaryotes need to be examined to understand their contributions to anaerobic processes in future studies.

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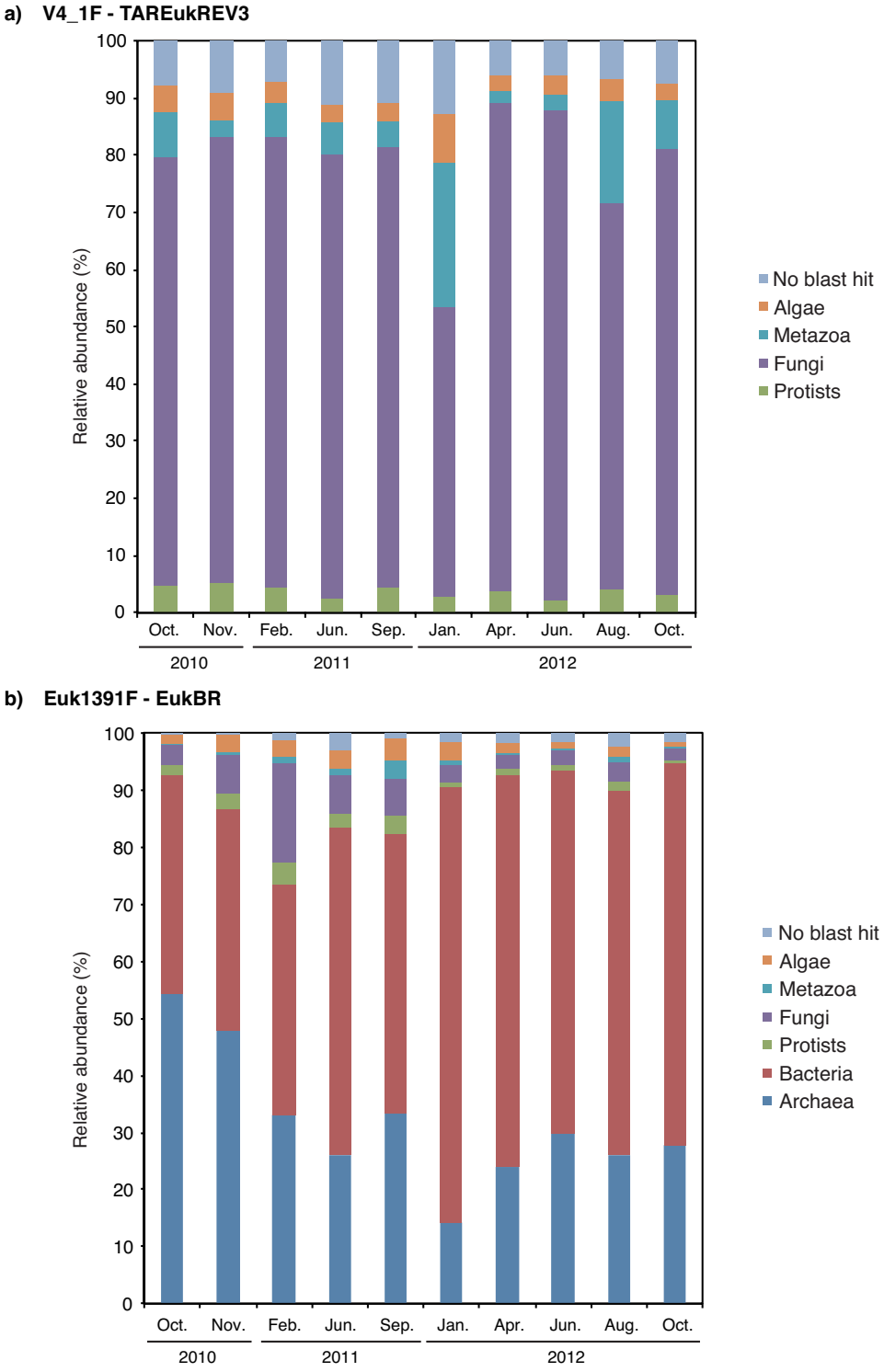
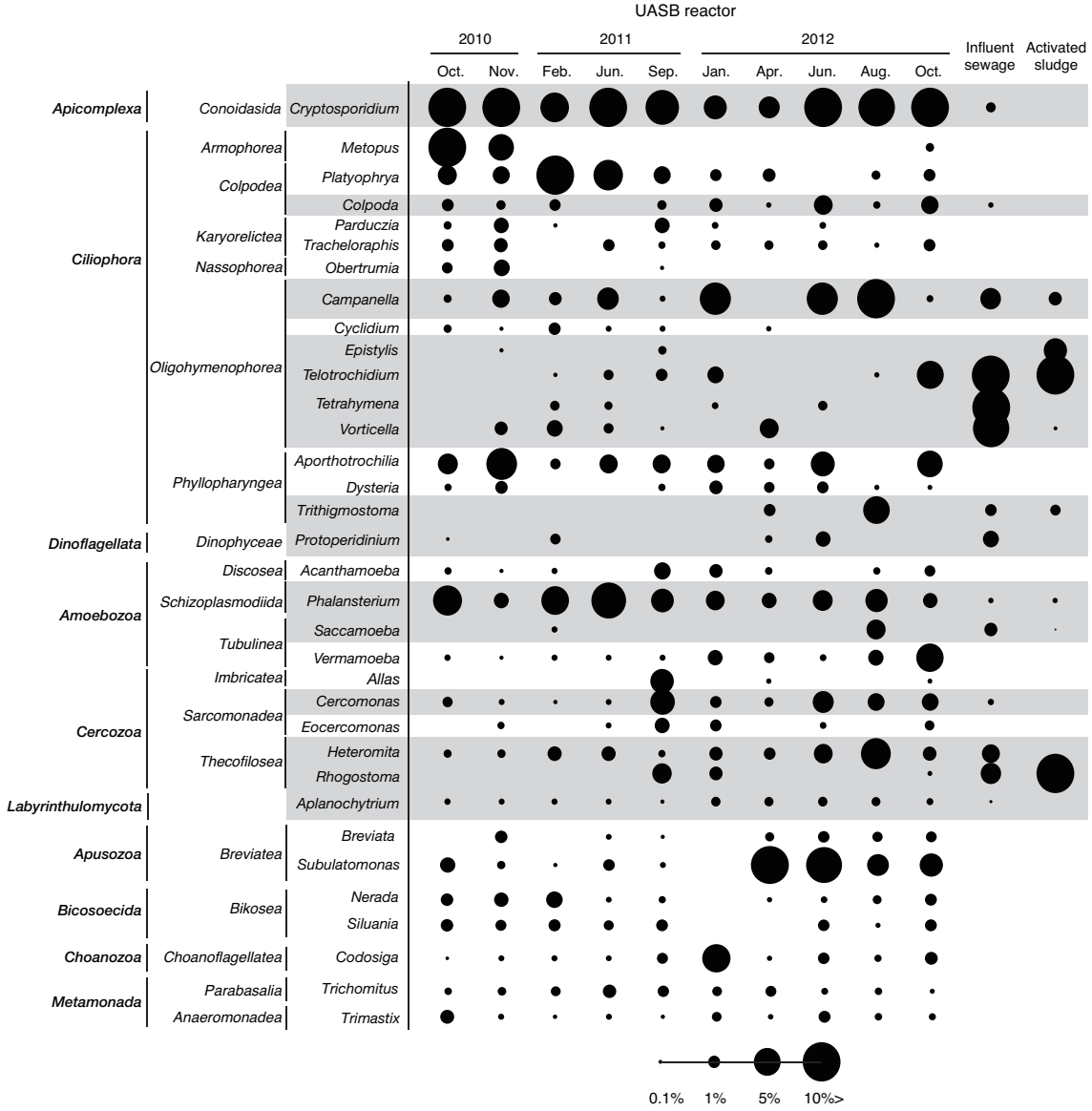
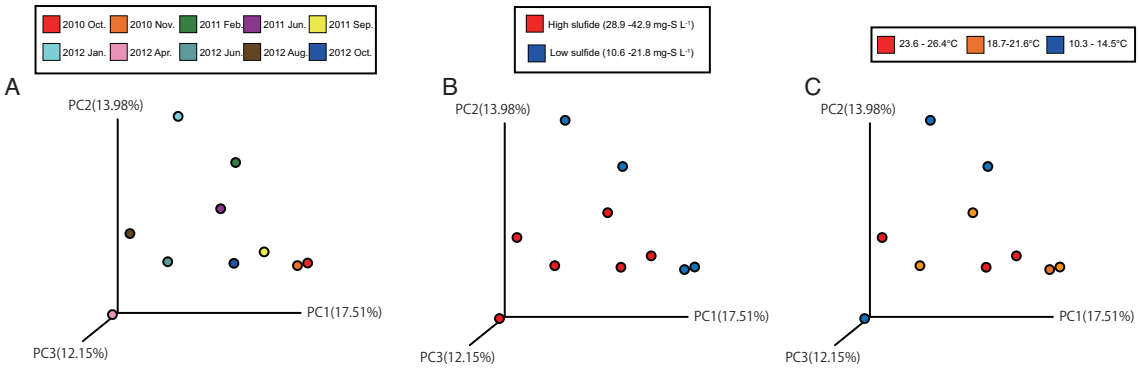
3.7 Supplementary Information



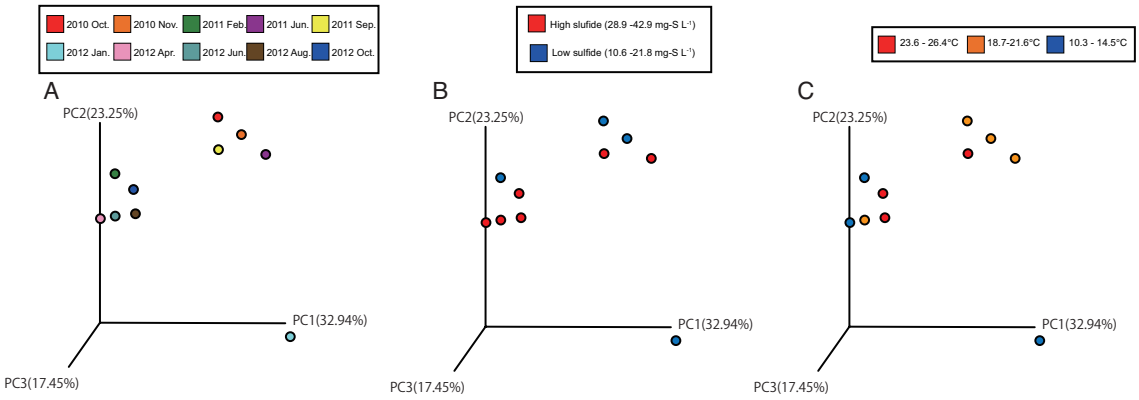
Supplementary Figure S3-1. Relative abundance of a) V4 and b) V9 region amplicons assigned to kingdom or domain level in the UASB reactor. Sequence reads that are not classified into any known group were labeled as "No blast hit."



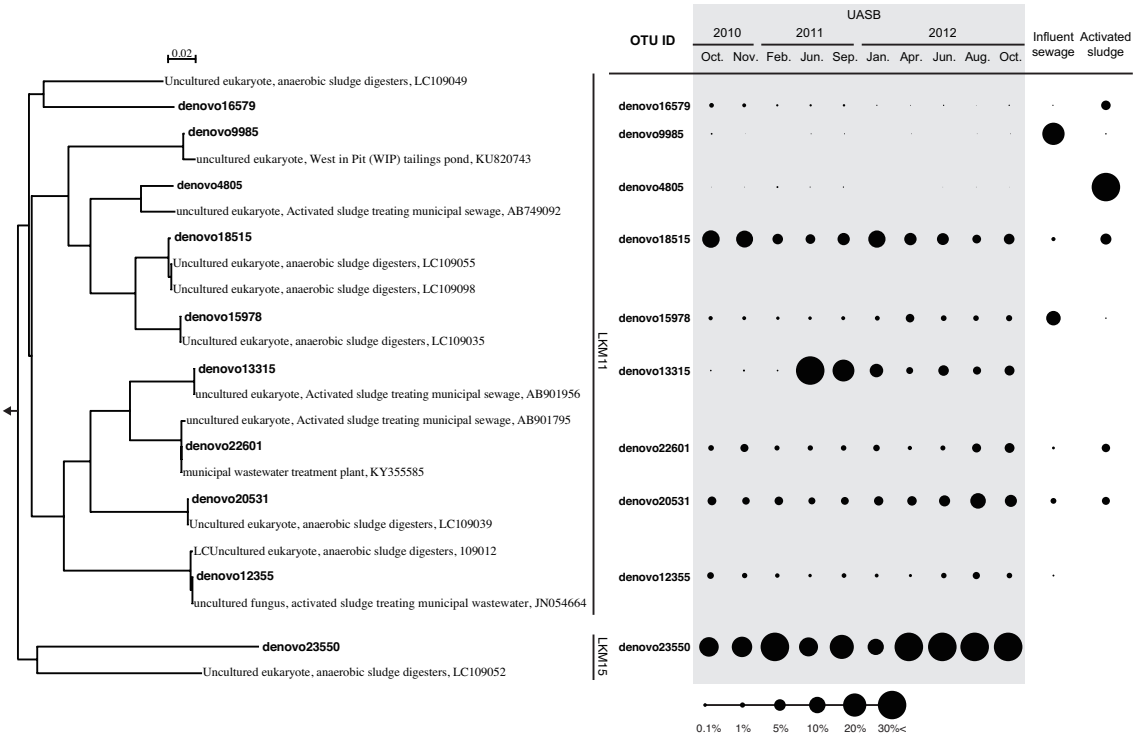
Supplementary Figure S3-2. Relative abundance of V4 region amplicons assigned to protist genus level in all samples from the UASB reactor, influent sewage, and activated sludge. Protist genera (represented by the areas of dots) representing at least 0.3% mean relative abundance per sample are shown. The size of each dot indicates the percentage of protist genera within the protist sequences. The gray colored part indicates shared protist genera in the UASB reactor, influent sewage, and activated sludge.



Supplementary Figure S3-3. Principal coordinates analysis (PCoA) based on Bray-Curtis distance of the protist OTU of the UASB reactor. Data colored by sampling date (A), reduced sulfate (sulfide) level (B), and temperature (C).



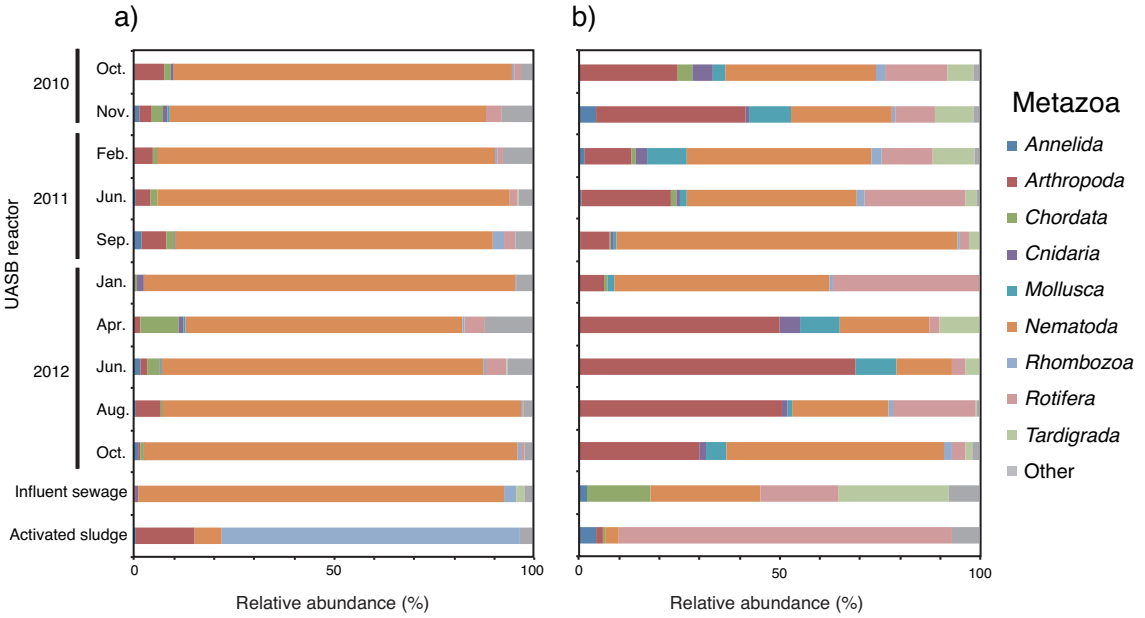
Supplementary Figure S3-4. Principal coordinates analysis (PCoA) based on Bray-Curtis distance of the fungal OTU of the UASB reactor. Data colored by sampling date (A), reduced sulfate (sulfide) level (B), and temperature (C).



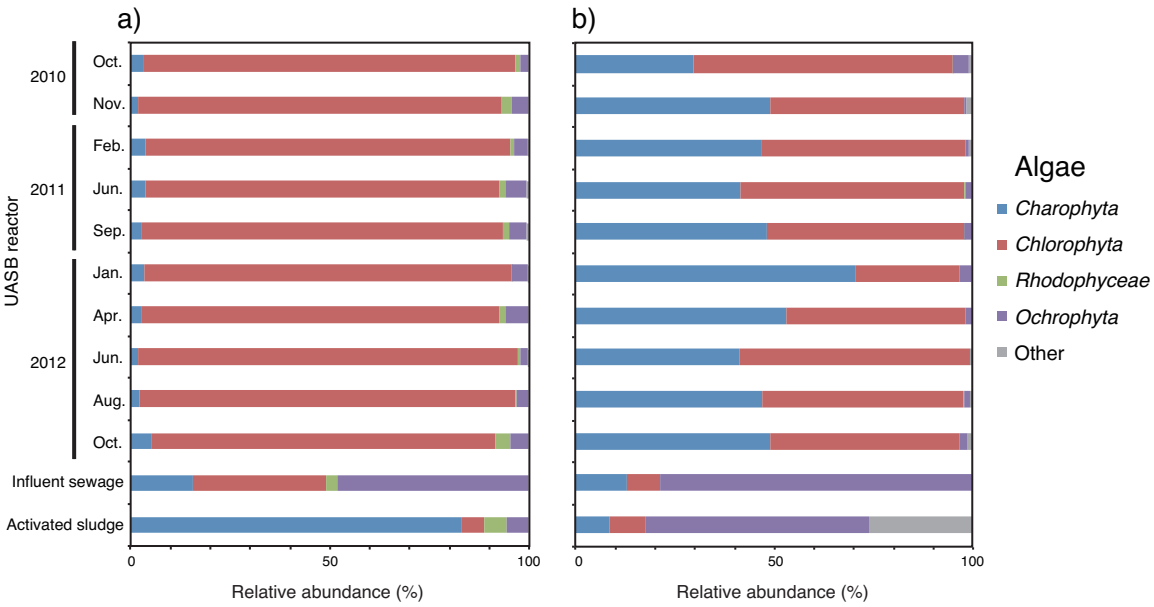
Supplementary Figure S3-5. A phylogenetic tree based on neighbor-joining (NJ) methods and relative abundance of OTUs assigned to uncultured LKM11 and LKM15 groups in phyla Cryptomycota, using the 18S rRNA gene. The 18S rRNA gene of Diplogaster sp. (GenBank accession number FJ516756) was used as an out-group (not shown). The OTUs obtained in this study are shown in bold type in the tree. The OTUs (represented by the areas of dots) representing at least 1.0% mean relative abundance per sample are shown. The size of each dot indicates the percentage of OTUs within the fungal sequences.

Sequences of V9 region specific primer pair		Euk1391f		EukBr	
	5' -	G T A C A C A C C G C C C G T C	- 3'	5' - G T A G G T G A A C C T G C A G A A G G A T C A	- 3'
Detected and dominant prokaryotes					
Pseudomonas poae (JQ045817)	5' - T	G T A C A C A C C G C C C G T C A C - - - - - - - - - - C C		G T A G G G G A A C C T G C G G A T G G A T C A	C - 3'
Arcobacter suis (MG195900)	5' - T	G T A C T C A C C G C C C G T C A C - - - - - - - - - - C C		G T A G G A G A A C C T G C G G T T G G A T C A	C - 3'
Syntrophobacter fumaroxidans (NR_075002)	5' - T	G T A C A C A C C G C C C G T C A C - - - - - - - - - - C C		G T A G G G G A A C C T G C G G C T G G A T C A	C - 3'
Candidatus *Methanomassiliicoccus intestinalis* (CP005934)	5' - T	G C A C A C A C C G C C C G T C A A - - - - - - - - - - C T		G T A G G G G A A C C T G C A G A T G G A T C A	C - 3'
Arcobacter bivalviorum (MG195891)	5' - T	G T A C T C A C C G C C C G T C A C - - - - - - - - - - C C		G T A G G T G A A C C T G C G G T T G G A T C A	C - 3'
Methanobacterium bryantii (AF028688)	5' - T	G C A C A C A C C G C C C G T C A C - - - - - - - - - - C C		G T A G G G G A A C C T G C G G C T G G A T C A	C - 3'
Methanobacterium subterraneum (NR_028247)	5' - T	G C A C A C A C C G C C C G T C A C - - - - - - - - - - C C		G T A G G G G A A C C T G C G G C T G G A T C A	- - 3'
Acinetobacter sp. (FJ193683)	5' - T	G T A C A C A C C G C C C G T C A C - - - - - - - - - - C C		G T A G G G G A A C C T G C G G A T G G A T C A	C - 3'
Dickeya chrysanthemi (KT862103)	5' - T	G T A C A C A C C G C C C G T C A C - - - - - - - - - - C C		G T A G G G G A A C C T G C G G A A G G A T C A	C - 3'
Pedomicrobium manganicum (X97691)	5' - T	G T A C A C A C C G C C C G T C A C - - - - - - - - - - C C		G T A G G G G A A C C T G C G G A T G G A T C A	A - 3'

Supplementary Figure S3-6. Sequence alignment of V9-specific primer pair and prokaryotic sequences detected in this study. Prokaryotic sequences were obtained from the NCBI GenBank database. Accession numbers of each prokaryotic sequence are shown in parentheses. The outline characters and black characters indicate matches and mismatches in the alignment, respectively.



Supplementary Figure S3-7. Relative abundance of a) V9 and b) V4 region amplicons assigned to Metazoa phylum level in all samples from the UASB reactor, influent sewage, and activated sludge



Supplementary Figure S3-8. Relative abundance of a) V4 and b) V9 region amplicons assigned to Algae phylum level in all samples from the UASB reactor, influent sewage, and activated sludge

Supplementary Table S3-1. Number of raw sequence reads and post QC sequence reads

Samples		V4					V9				
		Raw sequences	After QC*	After assembly	non-chmeric	OTUs	Raw sequences	After QC*	After assembly	non-chmeric	OTUs
2010	Oct.	44873	40092	23119	22742	4342	71112	68908	62796	62735	1356
	Nov.	35723	30305	16010	15609	4141	54145	52851	48424	48385	1357
2011	Feb.	35025	30883	18351	18060	3110	28450	27581	25431	25404	1042
	Jun.	30551	26693	16787	16537	2966	39604	38412	35410	35400	1234
	Sep.	37367	32203	18862	18472	4095	35459	34113	31356	31354	1138
2012	Jan.	30420	24427	11761	11554	2733	19937	19202	15448	15435	1020
	Apr.	25133	22435	14185	14020	2205	24792	23971	19827	19822	929
	Jun.	26277	24197	15761	15583	2360	20766	20171	16900	16893	809
	Aug.	29707	25655	13784	13604	2656	31287	30172	24970	24957	1206
	Oct.	39014	34249	20784	20412	3840	38763	37618	31192	31181	1078
Influent sewage		40959	17288	7160	7075	1400	16695	16550	15453	15429	663
Activated sludge		54805	23916	7174	7010	1377	17125	16497	13060	13059	697
Total		429854	332343	183738	180678	23908	398135	386046	340267	340054	4772

*QC: quality control

Supplementary Table S3-2. SSU rRNA gene data and alpha‐diversity indices of eukaryotic communities resulting from V4 and V9 regions of 18S rRNA gene sequencing

		Sequence number					Alpha - diversity indices of eukaryotes*					
V9		Total reads	Eukaryotes	Archaea	Bacteria	No blast hit	Observed species	Simpson	Shannon	Chao1	ACE	Goods_coverage
2010	Oct.	35187	4998	9395	20697	97	161	0.94	3.81	291	348	0.96
	Nov.	62511	4132	34684	23628	67	143	0.90	3.51	247	286	0.95
2011	Feb.	48796	6350	23761	18571	114	143	0.90	3.52	404	364	0.96
	Jun.	25258	6714	8636	9848	60	132	0.73	2.76	368	436	0.97
	Sep.	31131	5444	10622	14983	82	151	0.93	3.68	298	334	0.97
2012	Jan.	15746	1220	2389	11560	577	170	0.95	3.91	443	473	0.87
	Apr.	20176	1081	4994	13419	682	159	0.96	4.07	337	350	0.89
	Jun.	17067	766	5262	10621	418	155	0.92	3.71	327	389	0.86
	Aug.	25477	1788	6794	15784	1111	182	0.95	4.05	507	542	0.91
	Oct.	31600	1109	9395	20471	917	157	0.92	3.82	238	270	0.91
Influent sewage		15812	1593	47	13454	718	123	0.90	3.26	286	331	0.93
Activated sludge		14384	11765	0	807	1812	62	0.67	2.01	121	119	0.99
Total		340054	46286	115750	173389	4629						
V4												
2010	Oct.	22742	20940	0	5	1797	247	0.97	4.49	821	988	0.85
	Nov.	15609	14171	0	3	1435	310	0.96	4.64	1517	1448	0.79
2011	Feb.	18060	16781	0	7	1272	211	0.88	3.70	888	1090	0.87
	Jun.	16537	14685	0	4	1848	196	0.84	3.37	1243	1150	0.86
	Sep.	18472	16460	0	6	2006	251	0.93	4.10	1298	1165	0.83
2012	Jan.	11554	10089	0	2	1463	254	0.96	4.36	1279	1248	0.83
	Apr.	14020	13146	0	3	871	149	0.69	2.58	809	938	0.88
	Jun.	15583	14633	0	2	948	170	0.76	2.92	1200	1462	0.89
	Aug.	13604	12695	0	5	904	212	0.88	3.61	1239	1615	0.85
	Oct.	20412	18916	0	5	1491	241	0.92	4.04	1726	1383	0.86
Influent sewage		7075	5891	0	8	1176	199	0.90	3.75	703	689	0.88
Activated sludge		7010	5513	0	3	1494	193	0.93	3.84	742	793	0.88
Total		180678	163920	0	53	16705						

*Calculation at a sampling depth of 700 reads.

Supplementary Table S3-3. The relative abundances of dominant prokaryotic OTUs in all prokaryotic sequences of the V9 amplicon library

OTUs*	Accession No.	Closely related species	UASB (N = 10)	Influent sewage	Activated sludge
denovo1429	JQ045817	*Pseudomonas poae*	0.1%	29.3%	0.1%
denovo3211	MG195900	*Arcobacter suis*	0.0%	18.5%	0.0%
denovo2044	NR_075002	*Syntrophobacter fumaroxidans*	14.7%	0.0%	0.0%
denovo428	CP005934	*Methanomassiliicoccus intestinalis*	12.6%	0.0%	0.0%
denovo3974	MG195891	*Arcobacter bivalviorum*	0.0%	8.0%	0.0%
denovo3298	AF028688	*Methanobacterium bryantii*	6.5%	0.0%	0.0%
denovo913	NR_028247	*Methanobacterium subterraneum*	5.1%	0.0%	0.0%
denovo3127	FJ193683	*Acinetobacter sp.*	0.1%	5.0%	0.1%
denovo694	KT862103	*Dickeya chrysanthemi*	0.6%	4.8%	0.1%
denovo4667	X97691	*Pedomicrobium manganicum*	4.2%	0.0%	0.0%

* Top 10 OTUs selected from all prokaryotic sequences of the V9 amplicon library among all samples

Supplementary Table S3-4. Alpha diversity indexes calculated from protist sequences.

Samples		V4							V9						
		sequences	OTUs	shannon[a]	simpson[a]	observed species[a]	Chao1[a]	ACE[a]	sequences	OTUs	shannon[b]	simpson[b]	observed species[b]	Chao1[b]	ACE[b]
2010	Oct.	1106	370	4.08	0.96	131	519	647	935	141.0	3.03	0.90	40	79	86
	Nov.	833	378	4.41	0.98	151	1120	962	1005	144	2.66	0.87	32	67	68
2011	Feb.	787	307	4.16	0.96	139	772	692	1444	171	3.05	0.91	28	57	104
	Jun.	428	211	4.23	0.96	154	1268	1515	1016	155	2.79	0.88	42	96	147
	Sep.	829	395	4.64	0.98	174	1150	1240	1089	141	2.85	0.90	38	66	87
2012	Jan.	324	204	4.87	0.99	188	713	891	177	70	3.27	0.94	45	123	131
	Apr.	517	201	3.69	0.90	127	443	706	239	61	2.87	0.90	33	52	72
	Jun.	324	156	4.45	0.98	148	555	559	132	46	2.90	0.88	38	147	176
	Aug.	546	283	4.51	0.97	167	724	835	362	87	3.11	0.91	41	104	137
	Oct.	611	285	4.53	0.98	152	479	583	200	53	2.81	0.89	38	173	133
Influent sewage		1510	426	4.33	0.97	138	499	452	1080	114	2.84	0.89	34	97	99
Activated sludge		3799	617	3.26	0.88	93	313	440	11285	171	1.80	0.70	19	45	75
Total		11614	3204						18964	691					

[a] Calculation at a sampling depth of 300 reads, [b] Calculation at a sampling depth of 100 reads

Supplementary Table S3-5. Operational conditions and performance of the UASB reactor treating domestic sewage

Year	Month	UASB reactor			Water quality of influent				Water quality of effluent			
		Temp.	pH	ORP	SO_4^{2-}	Sulfide	SS	COD	SO_4^{2-}	Sulfide	SS	COD
		°C		mV	mg-S L^{-1}	mg-S L^{-1}	mg L^{-1}	mg L^{-1}	mg-S L^{-1}	mg-S L^{-1}	mg L^{-1}	mg L^{-1}
2010	Oct.	21.6	6.8	−241	7.2	0.0	93.0	299.3	8.1	17.0	31.7	120.1
	Nov.	18.7	7.0	−246	86.4	1.3	76.4	288.8	78.1	21.8	27.6	144.7
	Feb.	10.3	7.0	−182	109.6	1.5	92.4	317.8	79.0	15.0	48.5	198.8
2011	Jun.	21.6	6.8	−211	35.7	1.6	128.8	341.9	10.0	36.1	41.6	164.5
	Sep.	25.6	6.8	−277	34.2	3.5	89.2	383.0	4.2	31.7	38.9	145.1
	Jan.	10.6	7.3	−175	54.1	2.5	71.2	314.7	47.6	10.6	29.6	188.0
	Apr.	14.5	7.1	−203	45.8	1.1	110.6	383.8	12.6	33.4	58.5	265.0
2012	Jun.	21.4	6.9	−234	58.1	1.6	103.1	384.3	2.3	38.5	38.7	238.1
	Aug.	26.4	6.8	−280	37.3	2.7	98.7	372.7	3.7	28.9	40.3	172.7
	Oct.	23.6	6.8	−262	54.0	2.7	117.4	377.4	1.4	42.9	47.4	229.6

Supplementary Table S3-6. Alpha diversity indexes calculated from fungi sequences.

Samples		V4							V9						
						observed							observed		
		sequences	OTUs	shannon[a]	simpson[a]	species[a]	Chao1[a]	ACE[a]	sequences	OTUs	shannon[b]	simpson[b]	species[b]	Chao1[b]	ACE[b]
2010	Oct.	17024	2729	4.35	0.95	308	1434	1247	2444	169	2.48	0.78	55	94	114
	Nov.	12141	2707	4.34	0.95	326	2321	1622	1966	156	2.17	0.66	54	104	123
2011	Feb.	14229	1792	3.28	0.82	235	1200	1505	3266	200	2.21	0.71	53	132	189
	Jun.	12835	1862	2.98	0.78	215	1022	1116	4565	180	1.50	0.48	42	135	159
	Sep.	14221	2652	3.70	0.90	272	1937	1519	1995	145	2.67	0.83	57	113	141
2012	Jan.	5834	1160	3.95	0.93	289	1711	1965	387	99	3.26	0.90	86	200	233
	Apr.	11978	1344	2.27	0.62	171	1315	985	434	94	3.00	0.85	73	141	176
	Jun.	13375	1579	2.67	0.71	191	976	1076	435	80	2.68	0.80	64	117	158
	Aug.	9207	1389	3.01	0.78	221	1250	1553	813	139	3.05	0.84	80	170	180
	Oct.	15926	2305	3.59	0.86	249	1046	1168	576	97	2.72	0.76	76	149	208
Influent sewage		3594	419	2.30	0.73	144	1233	1378	452	58	1.80	0.56	45	133	90
Activated sludge		1222	257	2.85	0.74	221	1187	1661	302	44	2.73	0.87	44	76	73
Total		131586	15109						17635	527					

[a] Calculation at a sampling depth of 1,000 reads, [b] Calculation at a sampling depth of 300 reads

Chapter IV

Fermentative metabolites and predation behavior of *Cyclidium* sp., *Paracercomonas* sp., and *Trichomitus* sp. isolated from anaerobic granular sludge

Hirakata *et al.* (2019) in prep.

4.1 Introduction

In Chapter III, 18S rRNA gene sequencing revealed that many kinds of anaerobic protists such as amoeba, ciliate, and flagellate were existing in the UASB reactor treating domestic sewage. However, most anaerobic protists were still uncultured, physiological characteristics of those have poorly understood. Anaerobic protists are known as the major predator of prokaryotes that influence abundance, structure and diversity of prokaryotic communities in various anaerobic environments such as lake, groundwater, rumen, and bioreactor (Hirakata et al., 2016; Biagini et al., 1998; Holmes et al., 2014; Massana et al., 1994; Santra et al., 2002; Saccà et al., 2009). Bacterivorous protists have different hunting characteristics and species-specific prey preferences, and each of these protists has its own ecological niche (Šimek and Chrzanowski, 1992, Martinez-Garcia et al., 2012; Jousset 2012). Therefore, predation behavior and food selectivity of each protist species need to be investigated in order to understand their role in the UASB reactor treating domestic sewage.

In wastewater treatment process, anaerobic protist could contribute sludge reduction by predation, in addition, degrade particulate organic matter including bacterial cell and supply metabolite like soluble organic matter to prokaryotes (Priya et al., 2007; Prabhakaran et al., 2016). Anaerobic protists have produced various metabolites through the fermentative metabolism of food bacteria; ethanol, fatty acids, hydrogen, and carbon dioxide were reported as major product (Goosen et al., 1990; Shinzato et al., 2007; Zimorski et al., 2019). Our previous study suggested that supply of metabolite by anaerobic protist increased abundance of anaerobic syntrophic bacteria that prefer fatty acids and resulted high microbial diversity (Hirakata et al., 2016). Thus, metabolites of anaerobic protist are also one of important factor that influence microbial community in anaerobic ecosystems.

In addition, some anaerobic protists harbor endosymbiotic methanogen in the cytoplasm (van Bruggen et al., 1983; Finlay and Fenchel, 1991; Hirakata et al., 2015). A number of studies inferred that these endosymbiotic methanogens closely associated metabolism of host protist by scavenging hydrogen produced by protist (Hackstein and Vogels, 1997; Gast et al., 2009; Nowack and Melkonian, 2010; Shinzato et al., 2018). However, metabolisms of anaerobic protist including endosymbiotic methanogen have not been demosntrated. For investigation of decomposition pathway of substrate, s isotope tracer techniques have been applied previously (Wintsche et al., 2018; Teh et al., 2009). Although ^{13}C tracer techniques have been applied to investigate metabolism of parasite protist (Chapman et al., 1985; Saunders et al., 2015; Creek et al., 2015), no study reported about anaerobic protist and methanogen.

In this Chapter, we report the establishment of monoxenic culture of ciliate *Cyclidium* sp. strain YH that harbor endosymbiotic methanogen, flagellate *Trichomitus* sp. strain YH and *Paracercomonas* sp. strain YH isolated from anaerobic granular sludge in a domestic wastewater treatment plant. In addition, their characteristics such as predation behavior, ability, and metaboilte were investigated. Ingestion and digestion of bacteria by three protists and methane production by endosymbiotic methanogen were

demonstrated by tracer experiment using green fluorescent protein (GFP) and stable carbon isotope of ^{13}C. In addition, effects of food bacteria on these protist growth and metabolites were also investigated by feed experiment using various food bacteria.

4.2 Materials and methods

4.2.1 Isolation and cultivation of protists

Anaerobic granular sludge containing protist cells were obtained from sampling port at bottom of the UASB reactor at a domestic sewage treatment center of Nagaoka City, Japan (Hirakata et al., 2019), and cultured anoxically at room temperature (25˚C) in ciliate mineral medium containing the following per L of solution: 0.125 g K_2HPO_4, 0.025 g NH_4Cl, 0.4 g NaCl, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.15 g KCl, 0.25 g $CaCl \cdot 2H_2O$, 1.26 g $NaHCO_3$, 0.5 g $Na_2S \cdot 9H_2O$, 0.5 g L-cysteine hydrochloride monohydrate, 1 mg resazurin sodium salt, 1 mL vitamin solution, and 1 mL trace element solution. The pH of the media was adjusted to 7.0 with 1N HCl or NaOH. Culture bottles were flushed with N_2/CO_2 (80:20) gas and closed with a butyl rubber stopper. *E. coli* strain K-12 was used as the food bacteria for cultivation of anaerobic protist. *E.coli* was grown overnight in M9 minimal medium (Sambrook et al., 1989) containing 10 mM of glucose and 0.03% Yeast Extract at 37 °C, with shaking at 200 r.p.m. The cells were harvested by centrifugation at 8000×g, washed three times with 0.1M PBS and resuspended in the ciliate mineral medium. For cultivation of protist, stigmasterol and ergosterol dissolved in ethanol was also added to culture media at concentration of 1 μg mL^{-1} each, as previously reported (Wagener and Pfennig, 1987). Monocultures of anaerobic protists were obtained by transferring individual cells to culture bottle of fresh ciliate mineral medium containing food bacteria (i.e. *E. coli* strain K-12) from enriched culture using MM-89 and IM-9B micromanipulators (Narishige, Tokyo, Japan). Subculturing of each protists was performed every two or three week.

4.2.2 Confirmation of protist feeding of bacterial cells by using GFP expressing *E.coli*

To confirm feeding of bacterial cells by protist, green fluorescent protein (GFP)-expressing *E. coli* was used as the food bacteria. The chemically competent E. coli TOP10 (Invitrogen) was transformed with the vector pUC18-GFP (Nippon Gene) following the procedure provided by Invitrogen. *E. coli* transformants were selected for by growth in Luria broth (LB) supplemented with ampicillin 100 mg ml^{-1}. The GFP-expressing *E. coli* cells were harvested by centrifugation at 8000 rpm, washed two times with 0.1M PBS and resuspended in the fresh ciliate mineral medium in culture bottle. Each protist cells were inoculated into this culture bottles. After incubation for 30 minutes, protists in the suspension were taken out and checked for ingested bacteria with a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

4.2.3 Tracer experiment using isotopically labelled *E. coli*

To investigate the metabolism of protists, each protists were incubated with ^{13}C-labelled *E. coli* as a food bacteria in culture bottles of 50 mL fresh ciliate mineral medium. In addition, culture bottles containing medium with 13C-labelled *E. coli* but no protist cells were incubated in parallel as the control. For food bacteria, *E.coli* was grown overnight in M9 minimal medium containing 10 mM of fully ^{13}C-labelled glucose (Wako Chemicals, Tokyo, Japan) as the sole carbon source at 37 °C, with shaking at 200 r.p.m (Haig et al., 2015). The ^{13}C-labelled *E. coli* cells were harvested by centrifugation at 8000 rpm, washed two times with 0.1M PBS and resuspended in the fresh ciliate mineral medium in culture bottle (ca. 10^8 cells mL^{-1}). The initial bacterial density was 5.0×10^8 cells mL^{-1}. Incubation in tripricate was carried out at 25˚C in the dark. At appropriate intervals, samples were taken out for measurement of the protist and bacterial number and fermentation products.

4.2.4 Feeding experiments of anaerobic protists on various food bacteria

Propionibacterium acnes (strain UasXy-5) and *Trichococcus flocculiformis* (strain UasXy-4) were isolated in our laboratory, and *Bacteroides luti* strain UasXn-3 (JCM 19020) was isolated from anaerobic granular sludge as described in our previous study (Hatamoto et al., 2014). *Bacteroides graminisolvens* (JCM 15093T), *Methanospirillum hungatei* (JCM10133), *Clostridium acetobutylicum* (JCM1419), and *Moorella thermoacetica* (JCM 9319) were purchased from Japan Collection of Microorganisms (JCM, RIKEN, Saitama, Japan). *Methanobacterium beijingense* strain 8-2 (DSM15999) was obtained from the German Resource Centre for Biological Material (DSMZ). *Escherichia coli* strain TOPO10 was purchased from Invitrogen Corp. In addition, *T. flocculiformis* that grew filamentous form with chain of coccoid cells was divided to single coccus cells by sonication and also used as the food bacteria (designated T. flocculiformis-b). These characteristics of each food bacteria for protist culture are shown in Table S4-1.

Theses microorganisms except for *E.coli* were cultivated anoxically at 37°C in anaerobic basal medium reported previously (Hatamoto et al., 2014) with the following substrate: 10 mM glucose and 0.03% yeast extract for *B. luti, B. graminisolvens, C. acetobutylicum, M. thermoacetica, C. acnes*, and *T. flocculiformis*; H_2/CO_2 (80/20, v/v) for *M. beijingense* and *M. hungatei*. As described above, *E.coli* was cultivated in M9 minimal medium containing 10 mM glucose at 37 °C, with shaking at 200 r.p.m.

The food bacterial cells were harvested by centrifugation at 8,000 rpm, washed two times with 0.1M PBS and resuspended in the fresh ciliate mineral medium in culture bottle. The initial cells concentration adjusted to 10^8 cells mL^{-1}. Each protist cells were inoculated into this culture bottles from monoculture. At appropriate intervals, samples were taken out for measurement of the protist and bacterial number and fermentation products. These experiments were repeated and subcultured at least three times for each culture per food bacteria.

4.2.5 Analytical procedures

The number of protists was determined manually by counting the numbers of ciliate cells in a Neubauer chamber (ERMA, Tokyo, Japan) under an IX71 light microscope (Olympus, Tokyo, Japan). For count of total bacterial concentrations, the samples were filtered on a black polycarbonate membrane after sonication; the membrane was air-dried and mounted with 4,6-diamidino-2-phenylindole (DAPI). The bacteria on DAPI-stained membranes were enumerated by counting the total number of blue fluorescing bacteria. At least three randomly selected visual fields were used for the counting.

Methane was determined by gas chromatography with flame ionization detector (GC-2014, Shimadzu). Concentrations of $^{13}CO_2$ and $^{13}CH_4$ were analyzed using GCMS-QP2010SE gas chromatograph (Shimadzu, Kyoto, Japan). The generation time, feeding rates, and methane production rates of each protist were calculated from time courses of protists and bacterial number and methane production during exponential growth phase.

After filtration of the samples through 0.2-μm-pore-size membranes (Advantec, Tokyo, Japan), the fermentation products were analyzed by capillary electrophoresis (Agilent 7100 Photal, Otsuka Electronics).

4.2.6 DNA Extraction, PCR Amplification and 18S rRNA gene sequencing

The genomic DNA was extracted from cultured cells using a FastDNA SPIN Kit for Soil (MP Biomedicals, Carlsbad, CA, USA). The DNA concentration was determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Amplifications of near-full-length eukaryotic 18S rRNA genes were performed using universal eukaryote specific primer pairs of EukA (5'-AAC CTG GTT GAT CCT GCC AGT-3') and EukB (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') (Miyaoka et al., 2017). Premix Ex Taq Hot Start Version (TaKaRa Bio Inc., Shiga, Japan) was used for PCR amplification and the conditions were as follows; 5 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C; and the final extension step 10 min at 72°C. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and the concentrations were measured using Qubit dsDNA HS assay kit in a Qubit fluorometer (Thermo Fisher Scientific, USA). After purification of PCR products were sequensed with EukA, Ek-555F (5'-AGT CTG GTG CCA GCA GCC GC-3') (Miyaoka et al., 2017) and EukB by using a 3730xl DNA Analyzer (Applied Biosystems, CA, USA).

The nucleic acid sequences obtained were aligned in Clustal W software and a phylogenetic tree was constructed in MEGA 6.06 software (Tamura et al., 2013) by means of the maximum likelihood (ML; Jones-Taylor-Thornton model), neighbor joining (NJ; Poisson model), maximum parsimony (MP; close neighbor interchange in the random-tree search algorithm), and unweighted pair group methods with an arithmetic mean (UPGMA; a maximal composite likelihood model) using the 16S rRNA gene of *Bacteroides graminisolvens* (GenBank accession number NR_041642) as an outgroup.

4.2.7 Fluorescence in situ hybridization (FISH)

Protists used for FISH analyses were cultured with CMV medium without resazurin to minimize the amount of autofluorescence (Takeshita et al., 2019). Protist cells were collected by centrifugation (1,000 × g) and fixed with 4% paraformaldehyde for 1 h at 4°C, followed by two washes with 10 mM PBS. Fixed protist cells were embedded in low-melting agarose (Sigma-Aldrich, Steinheim, Germany) in each well of a 10-well glass slide. After drying, the cells on slides were dehydrated in 50%, 80%, and 100% ethanol for 4, 2, 1 min, respectively and dried again. Then, the fixed cells were hybridized with the oligonucleotide probe Arc915 for methanogenic archaea (Raskin et al., 1994). The samples were counterstained with DAPI before observation under a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

4.2.8 Nucleotide Sequence Accession Numbers

Sequence data were deposited in the DDBJ nucleotide sequence database under accession numbers LC497866 to LC497868. We deposited all sequences in GenBank.

4.3 Results

4.3.1 Isolation of protist

As the results of isolation process, monoxenic cultures of three anaerobic protists that have different morphologically features were obtained from the UASB reactor treating domestic sewage. These protists were identified as *Cyclidium* sp. YH, *Paracercomonas* sp. YH, and *Trichomitus* sp. YH based on morphologically features as reported previously (Clarke et al., 1993; Bass et al., 2009; Krishnamurthy, 1967). *Cyclidium* sp. YH was ciliate, *Paracercomonas* sp. YH and *Trichomitus* sp. YH were flagellate. *Cyclidium* sp. YH and *Paracercomonas* sp. YH could grow with only food bacteria. However, growth of *Trichomitus* sp. YH required food bacteria with ergosterol and stigmasterol as the co-substrates. Phylogenetic affiliations were further analyzed by examining 18S rRNA gene sequences amplified from each protist cultures. The length and GC content of the 18S rRNA gene sequences in this study are as follows: *Paracercomonas* sp. YH, 1,700 bp, 45.71%; *Cyclidium* sp. YH, 1,673 bp, 44.47%; and *Trichomitus* sp. YH, 1,471 bp, 47.99%. The 18S rRNA gene sequences that were obtained from three protist cultures were affiliated with the phylum Ciliophora, Metamonada and Cercozoa, respectively (Fig. 4-1): the sequence similarity of the 18S rRNA gene to *Cyclidium porcatum* was 97% (accession number Z29517) and to an *Paracercomonas anaerobica* was 97% (AF411272), and to *Trichomitus batrachorum* was 94% (AF124610). In addition, the endosymbiotic methanogens in inside of *Cyclidium* sp. YH cells were confirmed by fluorescence in situ hybridization with universal archaeal probe (Fig. S4-1). The endosymbiotic methanogens were not detected from *Paracercomonas* sp. YH and *Trichomitus* sp. YH cells.

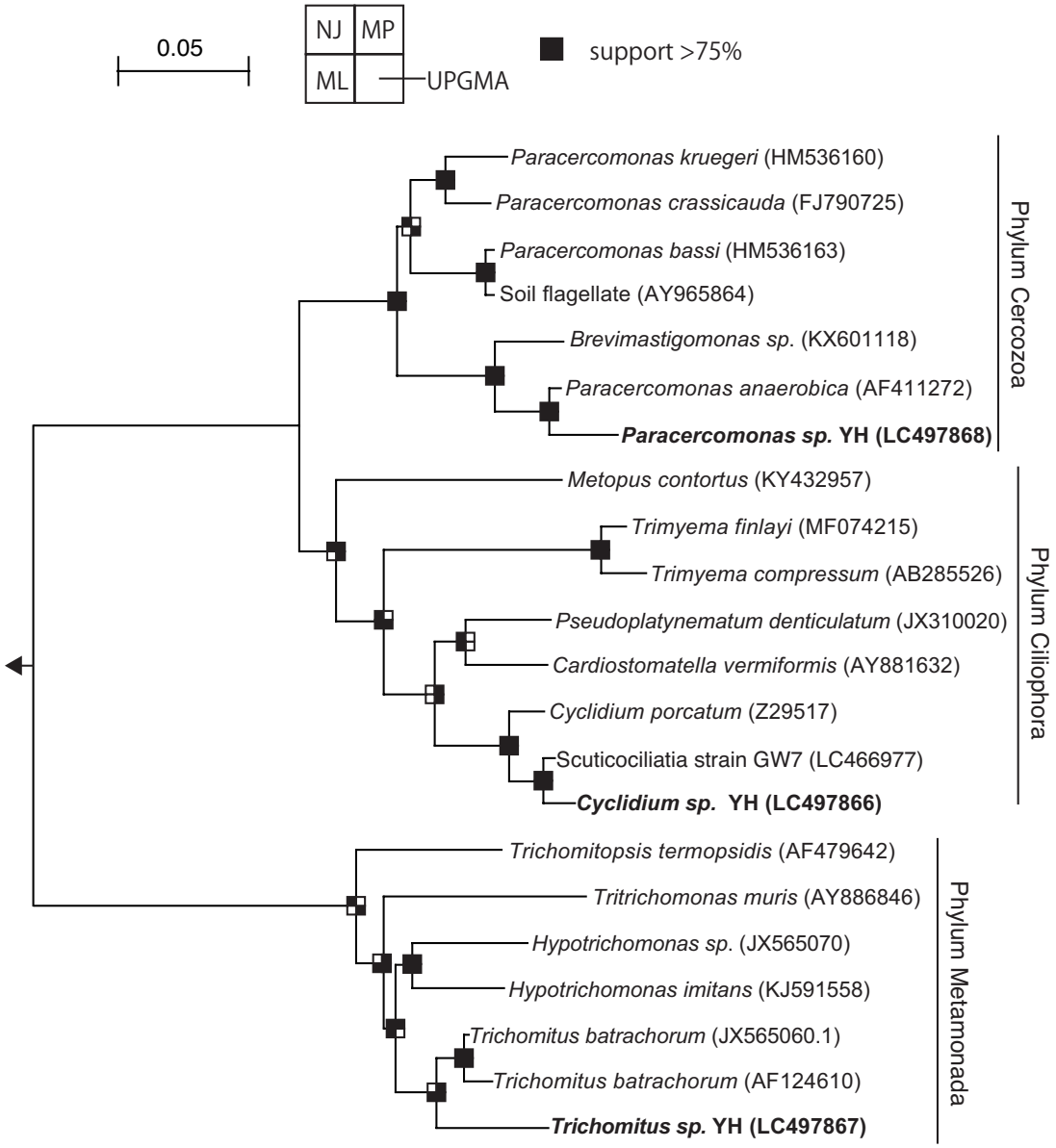


Figure 4-1. Neighbor-joining tree showing the phylogenetic affiliation of *Cyclidium* sp., *Paracercomonas* sp., and *Trichomitus* sp. isolated this study. Branching points that support a probability of >75% in the bootstrap analyses (based on 1,000 replications, estimated using the NJ method for the upper left sector, the MP method for the upper right sector, the ML method for bottom left sector, and the UPGMA method for the bottom right sector) are shown as black squares. The scale bars represent sequence divergence.

4.3.2 Confirmation of protist feeding of bacterial cells by using GFP expressing *E.coli*

To confirm feeding of bacterial cells by protist, GFP-expressing *E. coli* was used as the food bacteria. Internalization of GFP-expressing *E. col* in all protists cell were confirmed by a fluorescence microscope and showed that all protist species tested in present study ingested *E. coli* cell (Fig. 4-2). *Cyclidium* sp. YH seemed to ingest large number of bacterial cells than *Trichomitus* sp. YH and *Paracercomonas* sp. YH, because of the largest cell size of three protists. In addition, GFP-expressing *E. coli* cells ingested were localized in inside of *Cyclidium* sp. YH cell, suggesting that food vacuole formed in their cell. However, localizization of *E. coli* cells could not be observed in inside of *Trichomitus* sp. YH and *Paracercomonas* sp. YH cell.

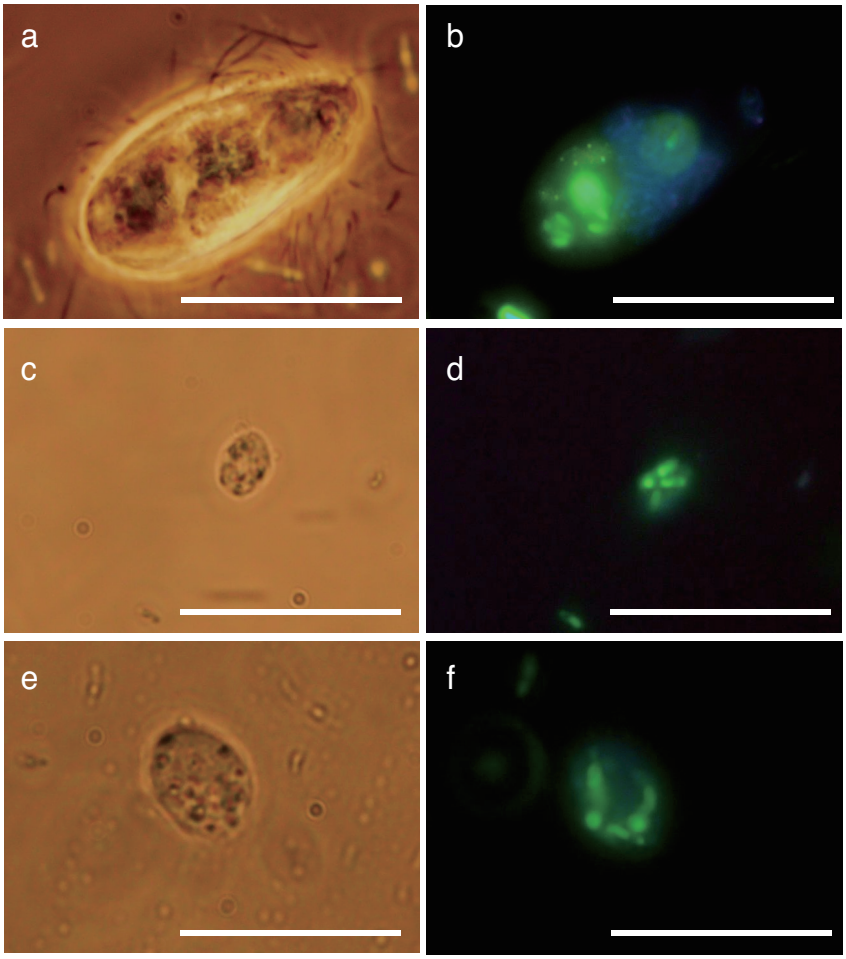


Figure 4-2. Internalization of GFP-*E.coli* by *Cyclidium* sp. (a and b), *Paracercomonas* sp. (c and d), and *Trichomitus* sp. (e and f). These images were obtained within 30 minutes after inoculation of GFP-*E.coli* to protists culture. Panels a, c, and e are bright field. Panels b, d, and f are fluorescence field. The scale bar represents 20 μm.

4.3.3 Feeding experiments using isotopically labelled *E. coli*

As shown in Figure 4-3, the time courses of each protists growth were different. After cultivation for 10 days, maximum number of *Trichomitus* sp. YH and *Paracercomonas* sp. YH reached to 1.2×10^4 cells mL^{-1} and 6.2×10^4 cells mL^{-1}, respectively. *Cyclidium* sp. YH grew to a maximum number of 2.2×10^3 cells mL^{-1} after 18 day of cultivation. The generation time of *Cyclidium* sp. YH, *Trichomitus* sp. YH and *Paracercomonas* sp. YH were 53.6 ± 13.6 h, 29.2 ± 5.4 h, and 35.5 ± 5.7 h, respectively. After exponential growth, the number of cells of each protist rapidly decreased, probably because accumulation of metabolites such as VFA to toxic levels in each culture (Narayanan et al., 2007). The number of bacteria decreased in all cultivation of protist than control. During exponential growth phase, ingestion rates of *Cyclidium* sp. YH, *Trichomitus* sp. YH and *Paracercomonas* sp. YH were 1.8×10^3, 1.2×10^2, and 0.6×10^2 cells per protist per hour, respectively.

The metabolite profiles in cultivation of each protists were clearly different (Fig. 4-4). In cultivation of *Cyclidium* sp. YH, major metabolite was acetate. Propionate, butyrate, and valerate were also detected in smaller amounts. On the other hands, acetate and butyrate were major metabolites of *Trichomitus* sp. with smaller amounts of propionate. In cultivation of *Paracercomonas* sp. YH, acetate and propionate were detected as major products. Lactate, formate, and ethanol were not detected in all cultivation. Hydrogen was increased in culture of *Trichomitus* sp. YH and *Paracercomonas* sp. YH. However, methane was detected instead of hydrogen in culture of *Cyclidium* sp. YH that harbor endosymbiotic methanogen. Acetate increased in culture of only food bacteria without protist, this is possibly due to autolysis of *E.coli* cells.

To examine digestion of ^{13}C-labelled *E. coli* by protist, $^{13}CO_2$ and $^{13}CH_4$ were measured in cultivation of protist (Table 4-1). After cultivation for 21 days, percentage of $^{13}CO_2$ increased in all cultivation of protist than control. The percentages of $^{13}CO_2$ were more abundance in order of cultivation of *Cyclidium* sp. YH, *Paracercomonas* sp. YH and *Trichomitus* sp. YH; this was resulted from amount of ^{13}C-labelled *E. coli* ingested by each protist. These results showed that ^{13}C-labeled *E.coli* was ingested and digested by protists. In addition, $^{13}CH_4$ was also detected in only cultivation of *Cyclidium* sp. YH. In case of *Cyclidium* sp. YH, it demonstrated that endosymbiotic methanogens convert CO_2 produced by protist to methane gas.

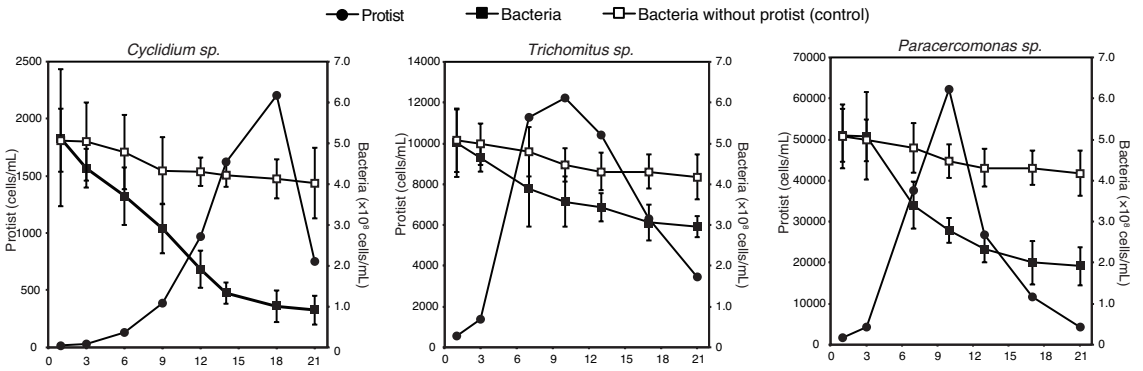


Figure 4-3. Growth of each protists with ^{13}C labeled *E. coli* as a food bacteria.

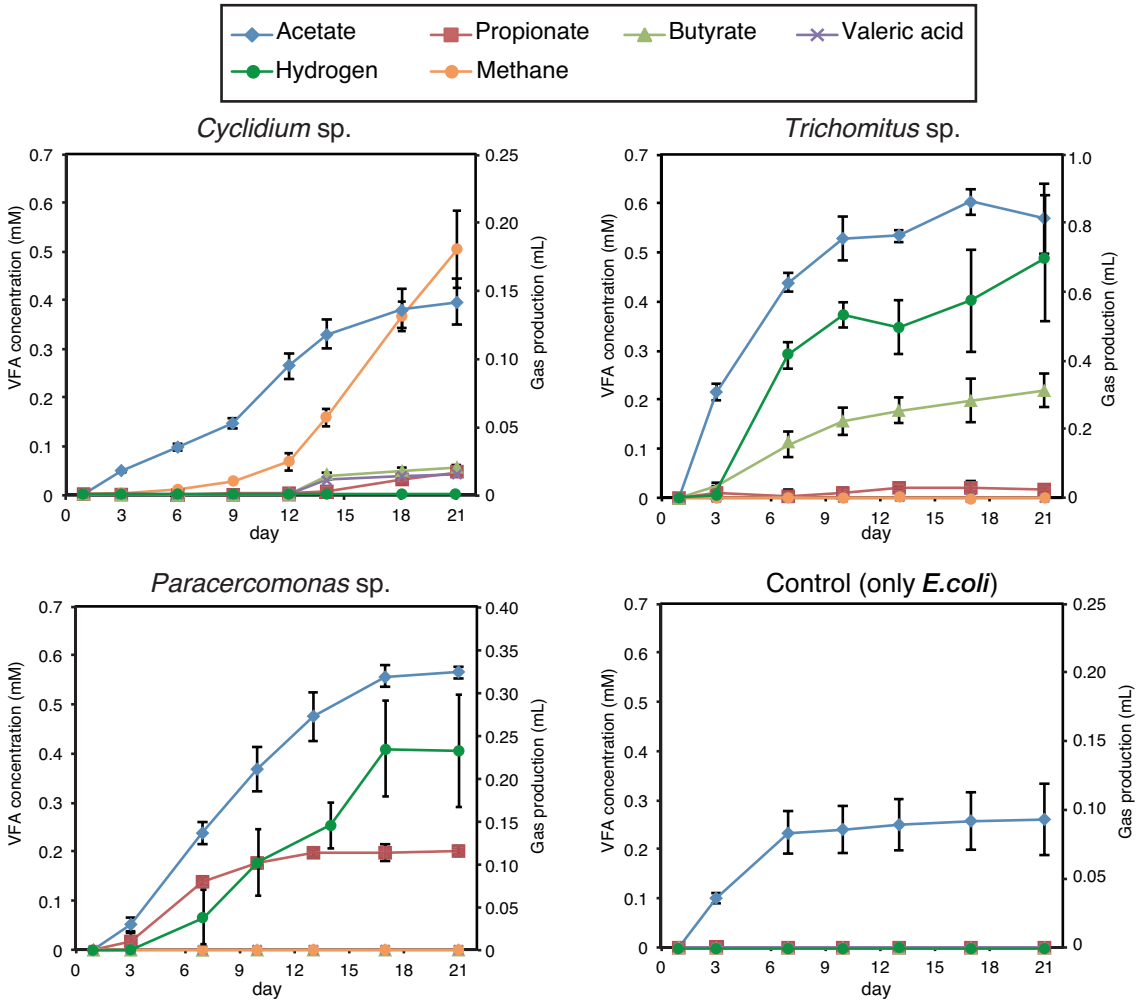


Figure 4-4. Production of fermentation products by each protists with ^{13}C labeled *E. coli* as a food bacteria.

Table 4-1. Percentage of $^{13}CO_2$ and $^{13}CH_4$ after cultivation.

Protist	$^{13}CO_2$ (%)	$^{13}CH_4$ (%)
Cyclidium sp.	7.0 ± 0.3	12.9 ± 2.3
Trichomitus sp.	3.9 ± 0.7	-
Paracercomonas sp.	4.6 ± 0.3	-
Control*	1.7 ± 0.1	-

-: no detected, **E.coli* only. (no protists)

4.3.4 Feeding behaviour of anaerobic protists

Table 4-2 shows the generation times of *Cyclidium* sp. YH, *Paracercomonas* sp. YH, and *Trichomitus* sp. YH during exponential growth phase cultured with various food bacteria. These three protists could not grow when archaea: *Methanobacterium beijingense* and *Methanospirillum hungatei* were used as substrate. The feeding experiments revealed that predation behavior of each protists on a range of bacteria were different. *Cyclidium* sp. YH could grow when all bacteria except for *T. flocculiformis* were used as the substrates. These results showed that *Cyclidium* sp. YH could ingest both gram-positive and gram-negative bacteria. However, no growth of *Cyclidium* sp. YH was observed on the culture with *T. flocculiformis*, suggesting that they can ingest rod and coccus cells but not filamentous form of bacteria. In contrast, *Trichomitus* sp. YH could ingest filamentous form of *T. flocculiformis.* Although *Trichomonas* sp. YH could grow regardless of gram staining properties of food bacteria, no growth was observed when *P. acnes* and *M. thermoacetica* were used. The growth of *Paracercomonas* sp. was observed on the feeding with only gram-negative bacteria: *E. coli*, *B. luti*, and *B. graminisolvens*. No growth of *Paracercomonas* sp. YH was observed when gram-positive bacteria

Table 4-2. Generation time of each protists fed various food bacteria and archaea.

Food	*Cyclidium sp.*	*Paracercomonas sp.*	*Trichomitus sp.*
E.coli	53.6 ± 13.6^{a}	35.5 ± 5.7^{a}	29.2 ± 5.4^{a}
Bacteroides luti	96.8 ± 14.4^{b}	33.3 ± 5.9^{a}	40.8 ± 9.5^{a}
Bacteroides graminisolvens	52.0 ± 8.7^{a}	27.1 ± 10.4^{a}	38.6 ± 15.0^{a}
Propionibacterium acnes	94.8 ± 15.5^{b}	-	-
Trichococcus flocculiformis	-	-	29.2 ± 9.6^{a}
Trichococcus flocculiformis -b	38.2 ± 4.2^{a}	-	31.4 ± 7.4^{a}
Moorella thermoacetica	53.2 ± 4.4^{a}	-	-
Clostridium acetobutylicum	49.1 ± 6.6^{a}	-	42.6 ± 9.8^{a}
Methanobacterium beijingense	-	-	-
Methanospirillum hungatei	-	-	-

-; not growth. Different superscript letters indicate ANOVA grouping with Tukey's test at 95% confidence.

were used as the food bacteria. This showed that cell-wall structure of bacteria could influence predation of *Paracercomonas* sp. YH.

The generation times of *Cyclidium* sp. YH were significantly different depending on food bacteria species ($p < 0.05$), even if they could grow. Among the food bacteria tested, generation times of *Cyclidium* sp. YH were faster on feeding with *E.coli*, *B. graminisolvens, Trichococcus flocculiformis*-b, *M. thermoacetica,* and *Clostridium acetobutylicum,* and slower on feeding with *B. luti* and *P. acnes*. Therefore, generation time of protists was influenced by food bacteria species. However, the generation times of *Trichomitus* sp. YH and *Paracercomonas* sp. YH were not different depending on food bacteria species tested in present study.

The ingestion rates during exponential growth phase cultured with various food bacteria were faster in the order of *Cyclidium* sp. YH, *Paracercomonas* sp. YH, and *Trichomitus* sp. YH (Fig. 4-5). The ingestion rates of *Cyclidium* sp. YH, *Paracercomonas* sp. YH, and *Trichomitus* sp. YH were 1.5-2.7 × 10^3, 1.3-1.5 × 10^2 and 0.4-0.6 × 10^2 cells·protist-1·hour-1, respectively. There were not significantly different for ingestion rates of each protist depending on food bacterial species in case of using bacteria that can be ingest by protist.

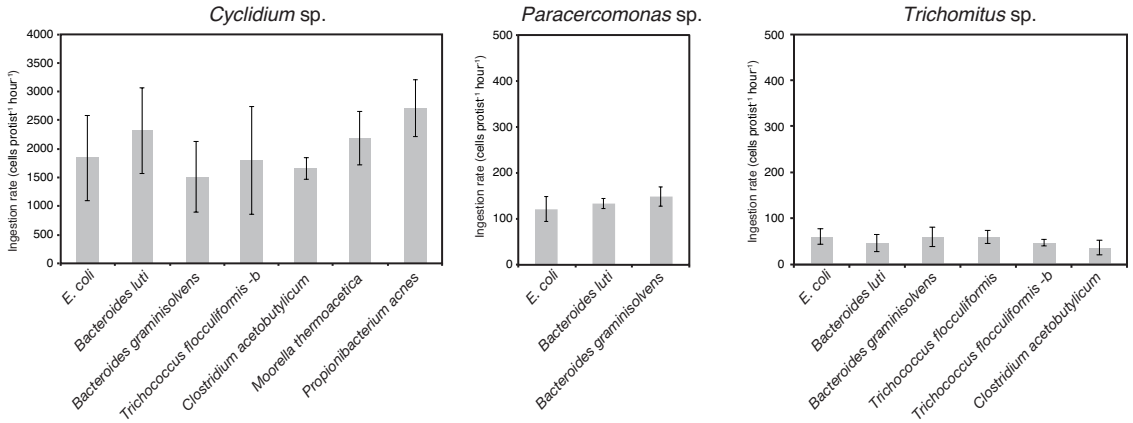


Figure 4-5. Ingestion rate of each protists fed various food bacteria during exponential growth phase.

The VFA compositions in cultivation of each protists varied markedly according to food bacteria species (Fig. 4-6). Although acetate was detected in all cultivation, propionate, butyrate, and valeric acid were not detected in some cases. In cultivation of *Cyclidium* sp. YH on feeding with *B. luti*, *B. graminisolvens,* and *M. thermoacetica*, acetate, propionate, butyrate, and valeric acid were detected as same as when *E. coli* was used as food bacteria. However, propionate was slightly or not detected when *T. flocculiformis*-b*, Clostridium acetobutylicum,* and *P. acnes* were used as food bacteria in cultivation of *Cyclidium* sp. YH. Furthermore, valeric acid was not detected in case of using *T. flocculiformis*-b and *P. acnes*, and butyrate was not detected in case of using *P. acnes*. Although major products were acetate, butyrate, and smaller amount of propionate in cultivation of *Trichomitus* sp. YH, butyrate and

propionate were not detected when *Clostridium acetobutylicum* was used as food bacteria. The major products in all culture of *Paracercomonas* sp. YH were acetate and propionate. Regardless of food bacteria species, hydrogen was detected in cultivation of *Trichomitus* sp. YH and *Paracercomonas* sp. YH, and methane was detected in culture of *Cyclidium* sp. YH (data not shown).

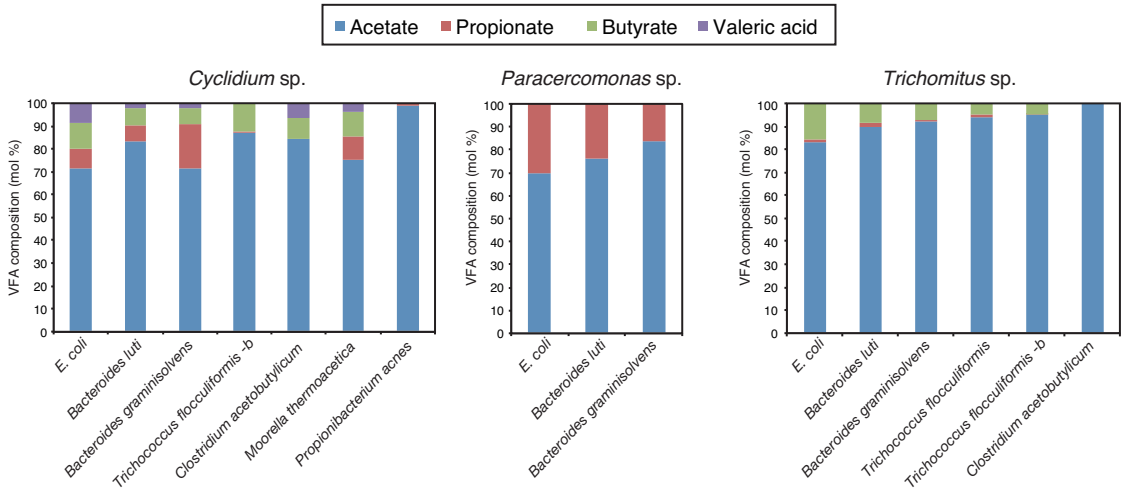


Figure 4-6. Fatty acid composition of each protists after cultivation with various food bacteria.

4.4 Discussion

In this study, we established monoxenic cultures of anaerobic protists *Cyclidium* sp. YH, *Paracercomonas* sp. YH, and *Trichomitus* sp. YH from a UASB reactor used to treat domestic sewage. These protist genera belong to the phyla Ciliophora, Cercozoa, and Metamonada, respectively, and are frequently found in anaerobic environments (Matsunaga et al., 2014; Triadó-Margarit and Casamayor, 2015). Although most studies have shown that anaerobic protists in these phyla are bacterivorous heterotrophic organisms in various environments (O'Kelly et al., 1999; Fenchel and Finlay, 1990; Takishita et al., 2007), very little is known about their specific roles in anaerobic reactors and natural ecosystems (Bayané and Guiot, 2011). In anaerobic granular sludge of UASB reactors, particularly, the ecological roles and functions of each anaerobic protist have not been characterized, despite the fact that these protist genera are frequently detected by 18S rRNA gene sequencing (Hirakata et al., 2019). Therefore, the present study provides important information of these protists' physiological characteristics such as generation time, ingestion rate, predation behavior, and metabolites.

Tracer experiments using GFP and ^{13}C-labeled *E. coli* showed that these three protists can ingest and digest bacterial cells. Members of the phyla Ciliophora and Cercozoa, which include our isolates *Cyclidium* sp. YH and *Paracercomonas* sp. YH, respectively, are well known predators of prokaryotes (Özen et al., 2018; Cunliffe and Murrell, 2010). The phylum Metamonada, to which *Trichomitus* sp. YH belongs, comprises both parasites and free-living protists. *Trichomitus batrachorum*, the species most closely related to our isolate *Trichomitus* sp.

YH, is an obligate commensal and parasitic protist found in the digestive tract of animals such as carabao, dogs, and pigs (Dimasuay et al., 2013). However, *Trichomitus* sp. YH demonstrated a free-living bacterivorous lifestyle, which is different from that described for *T. batrachorum*.

The three protists isolated from the UASB reactor have physiological characteristics that differ from one another. The rate of bacterial ingestion of *Cyclidium* sp. YH was the fastest of the three. *Cyclidium* sp. YH, which is a ciliate, was larger than the two other isolates, which are flagellates, suggesting that the difference in ingestion rates is associated with cell size. As a result of comparing the ingestion rates of 12 aerobic and 6 anaerobic protists species that similar to our experimental conditions (Table S4-2, Figure 4-7), it seems to logarithmically proportional to the cell volume regardless of either anaerobic or aerobic species. Our data and other anaerobic protist are in the same trends. The slope of regression line is 0.75 (Figure 4-7), which is close to slope of respiration rates of aerobic protists against cell volume (Fenchel and Finlay, 1983). Thus, we might be roughly estimate an ingestion rate based on protist cell size. Most ciliates have a large cell size and can consume more prokaryotic cells than flagellates (Meira et al., 2018; Gonzalez et al., 1990; Epstein and Shiaris, 1992). In particular, members of the genus *Cyclidium* have shown great efficiency in removing organic matter and can contribute to sludge reduction and treatment by predation in anaerobic treatment processes (Hirakata et al., 2019; Narayaman et al., 2016). Moreover, predation by protists strongly affect bacterial and archaeal community structures in anaerobic ecosystems (Ohene-Adjei et al., 2007; Murase et al., 2009). However, the predation ability of most anaerobic protists has not been reported. The ingestion rates of anaerobic protists are important considerations in determining the potential of various protists to contribute to sludge reduction in anaerobic ecosystems. In addition, flagellates reach higher cells numbers than ciliates (Gasol, 1993), as we found in this study. This suggests that *Trichomitus* sp. YH and *Paracercomonas* sp. YH can better contribute to control of bacterial populations than *Cyclidium* sp. YH in actual environments.

The generation time of flagellates was faster than that of the ciliate. Generation times of the ciliate *Cyclidium* sp. YH ranged from 38.2 to 96.8 h, which was similar to that of other anaerobic ciliates such as *Metopus contortus* (45–60 h), *Metopus palaeformis* (35 h), and *Plagiopyla frontata* (34 h), but faster than Scuticociliatia strain GW7 (112.8 h) and slower than *T. compressum* (10–33 h) (Fenchel and Finlay, 1990; Wagener and Pfennig, 1990; Holler and Pfennig, 1991; Takeshita et al., 2019). The generation times of the two isolated flagellates *Paracercomonas* sp. YH (27.1–35.5 h) and *Trichomitus* sp. YH (29.2–46.7 h) were also close to those previously reported for anaerobic flagellates such as *Psalteriomonas lanterna* (38 h) (Broers et al., 1993). However, the generation times of *Trichomitus* sp. YH were longer than the minimum generation times of 4–6 h for parasite species such as *Trichomonas vaginalis* in

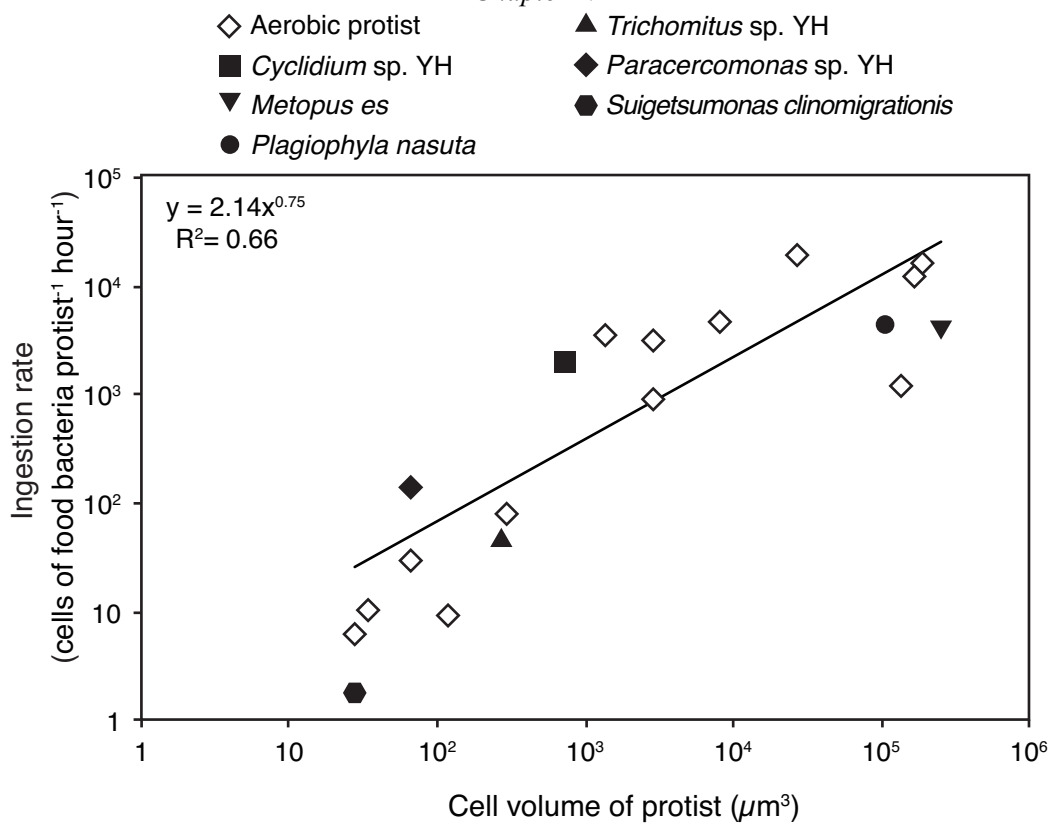


Figure 4-7. Ingestion rate of various protists under high prey concentration (10^7 cells mL^{-1} ≤) against cell volume. The plotted data is obtained this study and from the references listed in Table S2.

axenic culture (Nix et al., 1995). This could be because ingestion of bacterial cells by phagotrophy requires more energy than parasites' absorption of nutrients by osmotrophy.

Tracer experiments using ^{13}C-labelled *E. coli* showed that all protists ingested bacterial cells and digested them to produce CO_2 (Table 4-1). Hydrogen and CO_2 has been reported to be a common product of anaerobic protists (Yamada et al., 1994; Müller, 2003; Zimorski et al., 2019). Anaerobic ciliates and trichomonads have unique organelles, hydrogenosomes, instead of mitochondria, in which organic matter is oxidized to volatile fatty acids, hydrogen, and carbon dioxide for ATP synthesis (Müller. 1993). An anaerobic cercomonad (i.e, *Brevimastigomonas motovehiculus*) was also found to possess anaerobic mitochondrion-related (hydrogenosome-like) organelles (Gawryluk et al., 2016). Hydrogen and $^{13}CO_2$ were detected in the *Trichomitus* sp. YH and *Paracercomonas* sp. YH cultures, which suggested that these protists might also possess hydrogenosome-like organelles.

However, methane instead of hydrogen was detected in cultures of *Cyclidium* sp. YH (Fig. 4-4) because *Cyclidium* sp. YH harbors hydrogenotrophic methanogen inside its cells (Fig. S4-1). The presence of hydrogenosomes and methanogens in cells of the genus *Cyclidium* was observed previously (Esteban et al. 1993; Clarke et al., 1993). In addition, the present study showed that *Cyclidium* sp. YH directly supplied CO_2 and hydrogen to endosymbiotic

methanogens because both $^{13}CO_2$ and $^{13}CH_4$ were detected in a tracer experiment using ^{13}C-labelled *E. coli*. Many researchers have speculated that the relationship between host protist cells and endosymbiotic methanogens is based on syntrophic hydrogen and CO_2 transfer; however, this transfer has not been demonstrated previously (Goosen et al., 1990; Hirakata et al., 2015; Shinzato et al., 2007). This is the first study to show direct evidence of a symbiotic relationship between an anaerobic protist and endosymbiotic methanogens in a tracer experiment using a stable carbon isotope. However, the percentage $^{13}CH_4$ was low (12.9%), even though ^{13}C-labelled *E. coli* was used as the sole carbon source. This was probably because the endosymbiotic methanogens utilized bicarbonate in the medium and may indicate that the endosymbiotic methanogens used both CO_2 produced from hydrogenosomes and dissolved CO_2 from outside of host protist cells. Thus, endosymbiotic methanogens can contribute to the maintenance of hydrogen concentrations at very low levels by scavenging exterior hydrogen or that produced from hydrolysis.

Feeding experiments in this study showed that the feeding behaviors of the protists were affected by the cell shape and cell wall structure of food bacteria. The predation behavior of *Cyclidium* sp. YH was strongly affected by cell shape, because these cells ingested sonicated single coccus *T. flocculiformis*-b cells but not filamentous-form *T. flocculiformis* cells. Effects of bacterial cell shape on protist predation are well known; size-selective predation by protists exists, and filamentous bacteria are morphologically resistant to predation (Hahn et al., 1991; Corno and Jürgens, 2006). However, *Trichomitus* sp. YH was able to grow using both *T. flocculiformis* and *T. flocculiformis*-b as food bacteria. Some protists such as *Ochromonas* sp. and *Trochilioides recta* do have the ability to prey on filamentous bacteria (Wu et al., 2004; Bitton 2010). Our results showed that *Trichomitus* sp. YH may have similar predation ability. Furthermore, although *Trichomitus* sp. YH can take up both Gram-positive and -negative bacteria, *P. acnes* and *M. thermoacetica* did not support growth, suggesting that cell shape and cell wall structure of food bacteria were not the only factors associated with the growth of *Trichomitus* sp. YH. Yamada et al. (1994) also reported that food selectivity of the anaerobic ciliate *T. compressum* depends on the digestibility of food bacteria rather than on differences in general cell wall structure.

The feeding experiment with *Paracercomonas* sp. YH revealed, however, that the cell wall structure of food bacteria does influence feeding behavior. *Paracercomonas* sp. YH can grow only on Gram-negative bacteria. This might indicate that this protist does not have enzymes to digest the thick peptidoglycan layer of Gram-positive bacteria. None of the three protists grew when fed archaea. This also could be related to the indigestibility of their cell walls. Archaea possess cell walls like sheaths and pseudomurein as a cell wall component, which differs from the walls of bacterial cells. Digestive enzymes in protists should be examined in future studies.

In addition, we observed different predation behaviors of the three protists isolated in this study. Differences in the metabolites from each protist, such as propionate, butyrate, and valerate, also indicated differences in digestive pathways used by the organisms. These differences in predation behaviors are the result of the size, shape, and components of food bacterial cells and suggest that these three protists may play different roles or occupy different niches in anaerobic ecosystems.

Previous studies have suggested that food bacterial species influence fermentation patterns and growth rates of anaerobic protists such as *T. compressum* (Goosen et al. 1990; Holler and Pfennig 1991; Yamada et al., 1994). We also observed that the metabolites from and generation times of *Cyclidium* sp. YH changed depending on food bacteria. The metabolite profiles of *Trichomitus* sp. YH also changed depending on food bacterial species, although its generation times were not affected. Thus, our results support the idea that the metabolisms of anaerobic protists are affected by food bacterial species, and that anaerobic protists can supply various metabolites to environments based on the bacterial species ingested.

The generation times of *Cyclidium* sp. YH were affected by food bacteria, but its ingestion rates did not differ significantly based on food bacterial species. This suggests that the amount of nutrients obtained varies by food bacterium. To compare the relationship between each food bacterium and protist growth, we estimated the cell number and volume required for protist growth (Fig. S4-2). Significant differences were observed in the number and volume of cells need for *Cyclidium* sp. YH growth. Significantly more food bacterial cells or cell volume was needed for growth of *Cyclidium* sp. YH with *B. luti* and *P. acens* than *T. flocculiformis*-b. Therefore, it is likely that *T. flocculiformis*-b cells were better substrates for *Cyclidium* sp. YH than *B. luti* and *P. acens* for growth and a source of energy. This is consistent of cell yields (increased number of protist cells / number of ingested bacterial cells) for *Cyclidium* sp. YH. When *T. flocculiformis*-b used as food bacteria the cell yield was $1.1 \times 10^{-5} \pm 3.8 \times 10^{-6}$, this was significantly higher than that of *B. luti* ($2.8 \times 10^{-6} \pm 7.0 \times 10^{-7}$) or *P. acens* ($3.1 \times 10^{-6} \pm 3.1 \times 10^{-7}$) ($p < 0.05$). A previous study showed that use of specific bacteria as a substrate stimulated protist growth and maximum cell number (Caron et al., 1991; Odelson and Breznak, 1985). In addition, protists such as *Trimyema* sp., *Paramecium* sp., and *P. shumwayae* are known to need sterol and fatty acids as a growth factor (Wagener and Pfennig, 1987; Soldo and van Wagtendonk 1967; Skelton et al., 2008). In the present study, only *Trichomitus* sp. YH required ergosterol and stigmasterol for growth, which might be associated with food bacterial species and community structures in natural environments. Moreover, *T. compressum* cell yields were reported to change based on bacterial cell qualities like carbohydrate and protein contents (Holler and Pfennig, 1994). Although the metabolite composition of *Cyclidium* sp. YH with each food bacterium had no apparent association with fast or slow generation times, our results suggest that differences in the nutrients contained in food bacteria can affect protist growth. This

could be occurring in other protists species as well, because the metabolite compositions of *Trichomitus* sp. YH and *Paraceromonas* sp. YH were also altered depending on the food bacteria consumed. Further studies are required for elucidating the effects of different food bacteria on the growth of protists.

4.5 Summary of this chapter

In the present study, we isolated three bacterivorous protists representing different phyla from a UASB reactor used to treat domestic sewage. A tracer experiment using GFP and ^{13}C-labeled *E. coli* cells demonstrated ingestion and digestion of bacteria by these protists. In addition, the present study also showed that *Cyclidium* sp. YH directly supplied CO_2 and hydrogen to endosymbiotic methanogens. Moreover, our results showed that the physiological characteristics of these protists are clearly different. These reported generation times, ingestion rates, predation behaviors, and metabolite patterns provide important insights into the ecological roles of these protists in anaerobic ecosystems.

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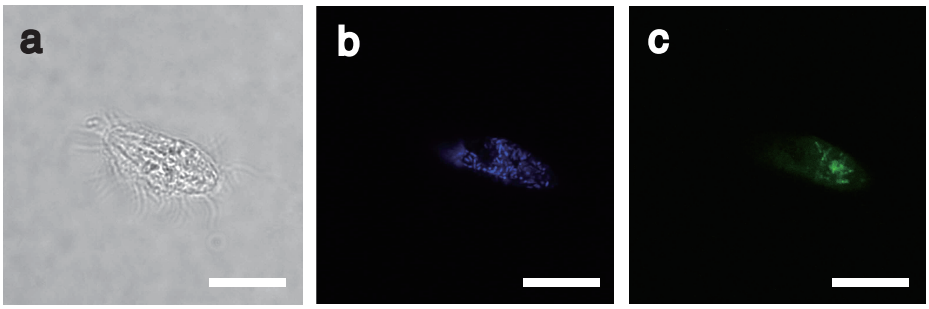
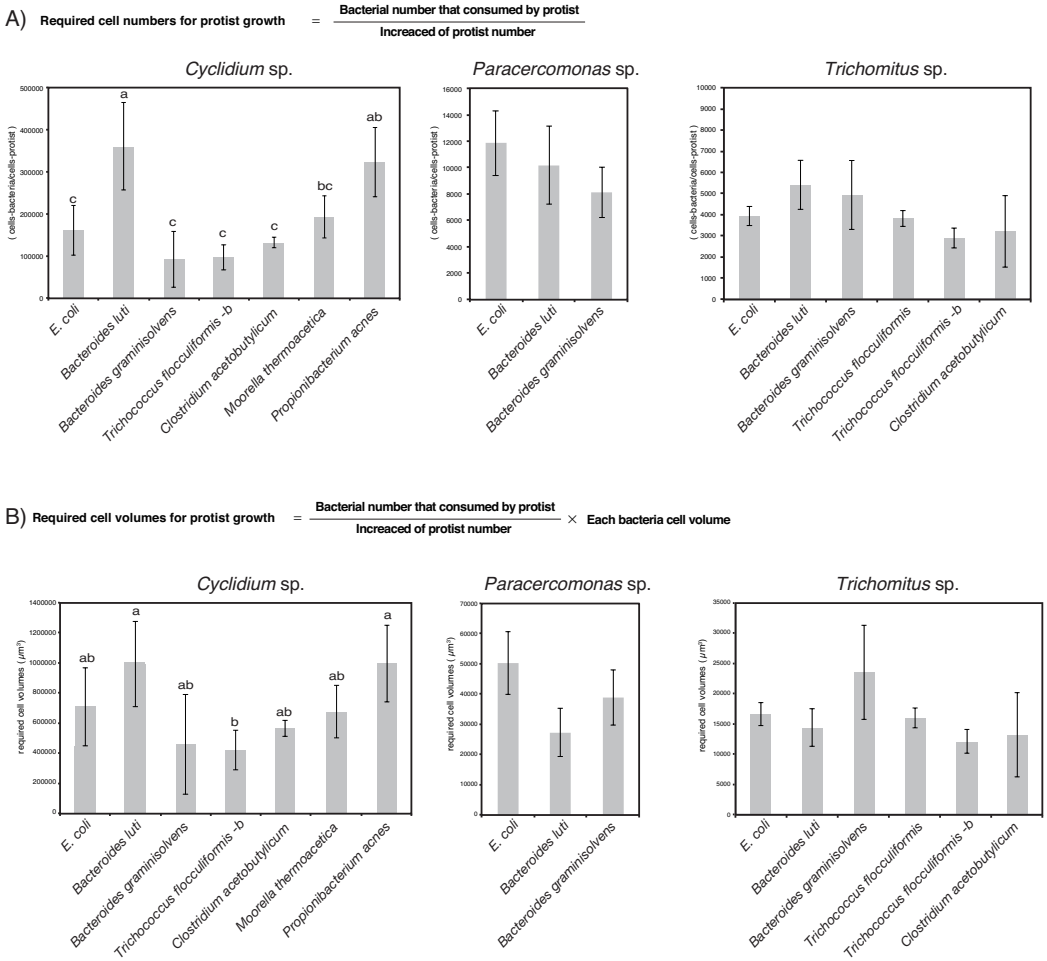
4.7 Supplementary Information



Supplementary Figure S4-1. Microscopic observations of Cyclidium sp.. a: Blight field. b: DAPI image. c: Fluorescent micrograph after hybridization with archaea-specific probe (Arc915) for the endosymbiotic methanogen of Cyclidium sp.. The scale bar represents 10 μm.



Supplementary Figure S4-2. Required cell number and volume for each protist growth estimated from increased of each protist number and decreased of food bacteria cell number during exponential growth phase, and food bacterial size such as wide, long, and diameter. Different letters indicate significantly different values ($P < 0.05$) by one-way ANOVA analysis.

Supplementary Table S4-1. Characteristics of food bacteria and archaea.

Species	Phylum	Gram-staining properties	Cell shape	Average cell size (μm wide and long)
Escherichia coli	Proteobacteria	negative	rod	1.0 - 3.0
Bacteroides luti	Bacteroidetes	negative	rod	0.5 - 1.4
Bacteroides graminisolvens	Bacteroidetes	negative	rod	0.5 - 2.9
Propionibacterium acnes	Actinobacteria	positive	rod	0.4 - 4.0
Moorella thermoacetica	Firmcutes	positive	rod	0.4 - 2.8
Clostridium acetobutylicum	Firmcutes	positive	rod	1.0 - 3.7
Trichococcus flocculiformis	Firmcutes	positive	filamentous	chains of coccoid cells (twenty to several hundred cells)
Trichococcus flocculiformis -b	Firmcutes	positive	coccus	2.3
Methanospirillum hungatei	*Euryarchaeota*	negative	curved rods	0.5 and 7.0 (form long chains of cells
Methanobacterium beijingense	*Euryarchaeota*	negative	rod	0.5 - 5.0

Supplementary Table S4-2. Maximum ingestion rates under high prey concentration (10^7 cells mL^{-1} ≤) and cell volume (μm^3) of protists.

species	Culture condition	group	Cell volume (μm^3)	Ingestion rate (cells of bacteria protist^{-1} h^{-1})	Ref
Cyclidium sp. YH	anaerobic	Ciliate	7.37×10^{2} [a]	2.00×10^{3}	this study
Trichomitus sp. YH	anaerobic	Flagellate	2.68×10^{2} [b]	0.52×10^{2}	
Paracercomonas sp. YH	anaerobic	Flagellate	0.65×10^{2} [b]	1.33×10^{2}	
Metopus es	anaerobic	Ciliate	2.54×10^{5} [a]	4.18×10^{3}	Massana et al., 1994
Plagiophyla nasuta	anaerobic	Ciliate	1.05×10^{5} [a]	4.30×10^{3}	
Suigetsumonas clinomigrationis	anaerobic	Flagellate	0.27×10^{2} [a]	1.8	Kondo and Okamura. 2017
Suigetsumonas linomigrationis	aerobic	Flagellate	0.27×10^{2} [a]	6.4	
Ochromonas sp.	aerobic	Flagellate	2.99×10^{2} [b]	0.78×10^{2}	Pfandl et al., 2004
Spumella sp.	aerobic	Flagellate	0.65×10^{2} [b]	0.29×10^{2}	
Bodo designis	aerobic	Flagellate	1.17×10^{2} [a]	9.1	Artolozaga et al., 2002
Jakoba libera	aerobic	Flagellate	0.65×10^{2} [b]	0.30×10^{2}	
Rhynchomonas nasuta	aerobic	Flagellate	0.33×10^{2} [b]	0.10×10^{2}	
Euplotes vannus	aerobic	Ciliate	1.70×10^{5} [a]	1.20×10^{4}	Tuorto and Taghon, 2014
Euplotes plicatum	aerobic	Ciliate	2.73×10^{4} [a]	1.94×10^{4}	
Uronema marinum	aerobic	Ciliate	1.36×10^{3} [a]	3.46×10^{3}	
Tetrahymena sp.	aerobic	Ciliate	7.96×10^{3} [a]	4.58×10^{3}	Eisenmann et al., 1998
Halteria sp.	aerobic	Ciliate	1.95×10^{3}	3.22×10^{3}	Sˇimek et al., 2000
Euplotes mutabilis	aerobic	Ciliate	1.88×10^{5} [a]	1.60×10^{4}	Zubkov and Sleigh, 1996
Stylonchia sp.	aerobic	Ciliate	1.35×10^{5} [a]	1.21×10^{3}	Hadas et al., 1998
Colpoda sp.	aerobic	Ciliate	2.85×10^{3} [a]	9.30×10^{2}	

a, estimated as ellipsoid body from length and width of each cell; b, estimated as sphere body from diameter of each cell.

Chapter V

Effects of predation by protists on prokaryotic community function, structure, and diversity in anaerobic granular sludge

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5.1 Introduction

Chapter IV described that predation behavior and metabolite of three anaerobic protists *Cyclidium* sp. (phylum Ciliophora), *Trichomitus* sp. (phylum Metamonada), and *Paracercomonas* sp. (phylum Cercozoa). Anaerobic protists have different predation behavior and selectivity of food bacteria depending on each species. In addition, these predation behaviors could contribute sludge reduction and change microbial community in the UASB reactor. Predation by protists is a major cause of prokaryotic community attrition in natural and artificial ecosystems (Pace, 1988) and modulates prokaryotic abundance and function, structure, and diversity. The impact of protist predation has been well documented in aerobic ecosystems (Pernthaler, 2005), in which protists consume up to 100% of prokaryotic standing stock per day (Anderson et a., 2012; 2013; Azam et al., 1983). Aerobic protists often show species-specific prey preferences (González et al., 1990; Šimek and Chrzanowski, 1992; Martinez-Garcia et al., 2012), which result in the disappearance of specific prokaryotes in ecosystems (Šimek et al., 2001; Murase et al., 2006; Rosenberg et al., 2009) as well as an increase or decrease in community diversity (Šimek et al., 1997; Pernthaler et al., 2001; Bell et al., 2010). Furthermore, predation by protists increases microbial activities including bacterial growth (Šimek et al., 1997; Bloem et al., 1988).

In contrast, little is known about the influence of protist predation on anoxic ecosystems (Massana and Pedrós-Alió, 1994; Schwarz and Frenzel, 2005; Oikonomou et al., 2014) except for the rumen. In the rumen, predation by protists increases or decreases the richness of archaeal species (Ohene-Adjei et al., 2007); one study showed that the predation influences prokaryotic abundance and methanogenic activities but not community diversity (Mosoni et al., 2011). The protists such as free-living ciliates are frequently found in anoxic freshwater (Massana and Pedrós-Alió, 1994; Bourland et al., 2014), marine sediments (van Bruggen et al., 1986; Esteban et al., 1994), landfill sites (Fenchel and Finlay, 1990; 1991), a rice field soil (Schwarz and Frenzel, 2005) and up-flow anaerobic sludge blanket (UASB) reactor (Hirakata et al., 2015). However, the impact of predation by these protists on prokaryotic community structure and on diversity in anoxic ecosystems, especially engineered ecosystems such as UASB reactor, has been poorly characterized.

Long-term cocultivation of ciliates and their prey (i.e. prokaryotic assemblages) is essential to investigate the influence of protist predation on anoxic ecosystems because rates of consumption of prokaryotic standing stocks are generally low in anoxic ecosystems (less than 0.1–6%) (Finlay et al., 1991; Massana and Pedrós-Alió, 1994; Saccà et al., 2009). For anoxic cultivation of ciliates, the batch cultivation method has been traditionally used (Wagener and Pfennig, 1987; Narayanan et al., 2007), but this method has an intrinsic problem: the predator-to-prey ratio changes transiently during the cultivation. Additionally, most of the studies on batch cultivation involved artificial substrates other than prokaryotic cells (e.g. wheat powder) to maintain the growth of ciliates (Narayanan et al., 2007), where the influence of predation by protists can not be assessed. To overcome these problems, a few research groups attempted to cultivate protists using the continuous cultivation

method (Simek et al., 1997; Bloem et al., 1988; Pernthaler et al., 2001) although no one succeeded in cultivating *Metopus* or *Caenomorpha* ciliates for >100 d in a continuous cultivation system.

The impact of predation by protists on prokaryotic community structure and diversity has been studied by DNA fingerprinting including denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism (Murase et al., 2006; Ohene-Adjei et al., 2007; Bell et al., 2010; Mosoni et al., 2011) and fluorescence in situ hybridization (Jürgens et al., 1999; Rosenberg et al., 2009; Gerea et al., 2013), but the resolution of these methods allows researchers to analyze differences only in major prokaryotic populations. Thus, the alterations of structure and diversity of a prokaryotic community that are induced by protist predation have not been assessed in detail so far. Recently, sequencing of 16S rRNA gene amplicons using Illumina MiSeq or HiSeq became the standard method for in-depth analysis of microbial-community structure because such analysis yields >10, 000 sequence reads per sample and allows researchers to examine the community structure in detail (Caporaso et al., 2010). These methods have recently been also applied to follow changes in prey communities upon predation (Baltar et al., 2015).

Consequently, our objectives in Chapter V were 1) to cocultivate anaerobic ciliates and prokaryotic assemblages in a continuous cultivation system and 2) to study the influence of predation by these protists on prokaryotic community function, structure, and diversity in anaerobic engineered ecosystems, in particular, in anUASB reactor. A UASB reactor that was packed with granular sludge (i.e. prokaryotic assemblages) was operated for 171 d on domestic sewage. Proliferation of *Metopus* and *Caenomorpha* ciliates was verified microscopically and by sequencing the 18S rRNA gene amplified by single-cell PCR. In order to assess the effects of predation by the *Metopus* or *Caenomorpha* ciliates, another UASB reactor served as a control reactor, where their proliferation was inhibited by cycloheximide, a specific growth inhibitor for eukaryotes. After 171 d of operation, sequencing of 16S rRNA gene amplicons using Illumina MiSeq was performed, and prokaryotic-community structure and diversity were compared between the two UASB reactors.

5.2 Materials and methods

5.2.1 Inoculums

Granular sludge was collected from a UASB reactor (1148 L; 4 m high, 0.56 m diameter) at a domestic wastewater treatment plant (Nagaoka, Japan). The reactor had been continuously and stably operated for more than five years on domestic sewage; the operational conditions and chemical oxygen demand (COD) removal efficiency were described previously (Takahashi et al., 2011). Ciliates could be detected in the inoculums by optical microscopy, and their abundance was less than 10 cells ml^{-1}.

5.2.2 Operation of laboratory scale UASB reactors

Two UASB reactors (500 ml; 6 cm in diameter and 30 cm high; Fig. 5-1) were operated for 171 d after inoculation with 400 ml of the granular sludge. The UASB reactors were operated with continuous feeding of domestic sewage collected from a domestic wastewater treatment plant

(Nagaoka, Japan). Typical composition of the sewage is shown in Table S5-1. Prior to use, the sewage was filtered through a polyethylene sieve (mesh size 350 μm) to remove large solid particles. Approximate loading rates of total COD were increased stepwise by shortening hydraulic retention time (HRT) as follows: 0.5 (g COD) L^{-1} d^{-1} with 10 h of HRT for 0–83 d (Run 1), 1.0 (g COD) L^{-1} d^{-1} with 5 h of HRT for 83–138 d (Run 2), 2.5 (g COD) L^{-1} d^{-1} with 2 h of HRT for 138–160 d (Run 3), and 4.0 (g COD) L^{-1} d^{-1} with 1.25 h of HRT for 160–171 d (Run 4). The reactors were operated in an isothermal room at 20 ± 2°C. Control of pH was not used during the operation, and pH of the influent and effluent was 7.1 ± 0.2 (average ± standard deviation) and 6.7 ± 0.2, respectively. The biogas generated in the UASB reactors was collected in a gas-sampling aluminum bag (Techno Quartz, Tokyo, Japan) installed on top of the reactors. The granular sludge was collected from a sampling port located 3 cm above the bottom of the reactors.

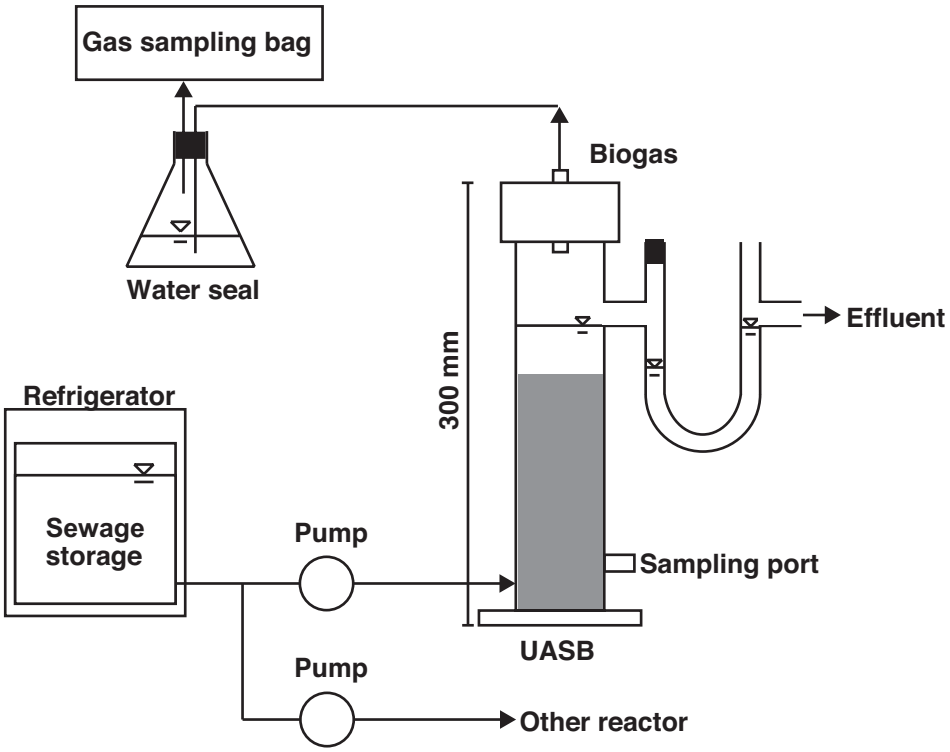

Figure 5-1. A schematic diagram of an up-flow anaerobic sludge blanket (UASB) reactor. Sewage was supplied from a single storage bottle to 2 UASB reactors (i.e., the coculture and control reactors) by peristaltic pumps. UASB reactor configuration were the same between the 2 reactors.

In one UASB reactor, proliferation of the protists was inhibited by addition of cycloheximide (1 g L^{-1}) at the start of operation (day 0) as described previously (Kota et al., 1999; Holmes et al., 2014). Cycloheximide is an antibiotic specific for eukaryotes and binds to the initiation factor of the eukaryotic 60S ribosomal subunit, thereby inhibiting protein synthesis in eukaryotic cells (Schneider-Poetsch et al.,

2012). Cycloheximide has been traditionally employed to prepare prokaryotic culture without eukaryotes (Kota et al., 1999; Holmes et al., 2014; Julian Schwarz and frenzel, 2005; Priya et al., 2007). Although growth inhibition of anaerobic bacteria by cycloheximide was previously reported (Tremaine and Mills, 1987; Ha et al., 1995), the addition of cycloheximide did not influence to bacterial and methanogenic activities in anaerobic reactor (Priya et al., 2007). Supplementation with cycloheximide was repeated from day 40 to day 70 of the operation every ten days (Run 1') because the protists were detectable in the control reactor on day 40 of the operation. The UASB reactors with and without the cycloheximide treatment are hereafter called control and coculture reactors, respectively.

5.2.3 Chemical analysis

The pH levels were measured using pH meter D-51 (Horiba, Kyoto, Japan). Total and soluble COD concentrations were determined using the HACH option 435 on a DR-2000 spectrophotometer (Hach, Tokyo, Japan) according to the manufacturer's instructions. For quantification of the soluble COD fraction, liquid samples were filtered through the GB-140 glass filter paper (pore size 0.45 μm; Advantec, Tokyo, Japan), and the filtrates were subjected to the COD measurement.

Suspended solids (SS) and retained sludge (mixed liquor suspended solids : MLSS) concentrations were determined in accordance with the standard method (APHA. 2012). Sludge volume in the UASB reactors was calculated from the retained sludge concentrations and sludge bed volume.

Gas composition was determined by injecting 1 mL of a gas sample into a GC-2014 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector and a molecular sieve-5A column (Shimadzu, Kyoto, Japan). Retention time of methane, hydrogen, and carbon dioxide gases was determined by analyzing standard gases purchased from GL Science (Tokyo, Japan). The volume of the gas that was collected in a gas sampling bag was determined by the liquid displacement method using 0.5N NaOH. Methanogenic activities were calculated from volumes of daily methane gas production and amounts of anaerobic granular sludge retained in UASB reactors.

5.2.4 Analysis of protozoan community structure

Abundance of ciliates was determined manually by counting the numbers of ciliate cells in a Neubauer chamber (ERMA, Tokyo, Japan) under an IX71 light microscope (Olympus, Tokyo, Japan). At least five randomly selected visual fields were used for the counting. *Metopus* and *Caenomorpha* ciliates were identified according to their morphological features as reported previously by Esteban et al. (1994) and Martin-Gonzalez et al. (1988), respectively. Phylogenetic affiliation of the ciliates was further ascertained by sequencing the 18S rRNA gene. After 171 d of operation, stand-alone cells of the *Metopus* and *Caenomorpha* ciliates were physically isolated by a MM-89 and IM-9B micromanipulators (Narishige, Tokyo, Japan). After several washes, each cell was transferred into a sterile PCR tube containing 3 μL of sterile distilled water. The cell was disrupted by three freeze-thaw cycles (i.e. freezing at -80°C and thawing at 60°C) and was directly subjected to PCR. Oligonucleotide

primers Euk-82F (5'-AAACTGCGAATGGCTC-3') and MedlinB (5'-TGATCCTTCTGCAGGTTCACCTAC-3') (Medlin et al., 1988; López-García et al., 2003) were used to amplify a region of the eukaryotic 18S rRNA gene. The PCR reaction mixture had a volume of 10 μL and contained the oligonucleotide primers (0.5 μM each), deoxynucleoside triphosphates (dNTPs; 200 μM each), 1× PCR buffer, and AmpliTaq Gold (0.025 U μl^{-1}; Thermo Fisher Scientific, Yokohama, Japan). The PCR was performed using a C1000 thermal cycler (Bio-Rad Laboratories, Benicia, CA, USA) under the following cycling conditions: 10 min at 95°C; 50 cycles of 45 s at 95°C, 45 s at 56°C, and 2 min at 72°C; and the final extension step 10 min at 72°C. The amplification of the 18S rRNA gene region was ascertained by agarose gel electrophoresis using a DNA Size Marker 4 (Nippongene, Tokyo, Japan). The amplicon was purified using a Gene Clean Turbo Kit (Qiagen, Hilden, Germany), and the DNA was sequenced by the Sanger method using a 3730xl DNA Analyzer (Thermo Fisher Scientific, Yokohama, Japan). The determined nucleic acid sequences were aligned in the Clustal W software and a phylogenetic tree was constructed in the MEGA 6.06 software (Tamura et al., 2013) by means of the maximum likelihood (ML; Jones-Taylor-Thornton model), neighbor joining (NJ; Poisson model), maximum parsimony (MP; close neighbor interchange in the random-tree search algorithm), and unweighted pair group methods with arithmetic mean (UPGMA; a maximal composite likelihood model) using the 18S rRNA gene of *Discophrya collini* (GenBank accession number L26446) as an outgroup.

5.2.5 Analysis of the bacterial- and archaeal-community structure

Genomic DNAs were extracted from the granular sludge using an ISOIL Beading Kit for Beads (Nippongene, Tokyo, Japan). Genomic DNA extraction was replicated from granular sludge in both reactors. Concentrations of the extracted DNAs were measured by means of the Picogreen dsDNA Quantification Kit (Thermo Fisher Scientific, Yokohama, Japan) and a Versafluor fluorometer (Bio-Rad Laboratories, Benicia, CA, USA). Amplification of the 16S rRNA gene region was performed using oligonucleotide primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') corresponding to the V4 region of the 16S rRNA gene (Caporaso et al., 2012). The PCR mixture had a volume of 50 μl and contained 50 ng of the extracted DNAs, the oligonucleotide primers (0.5 μM each), dNTPs (200 μM), 1×PCR buffer, and AmpliTaq Gold (0.025 U · μl^{-1}). The cycling conditions were as follows: 3 min at 94°C; 30 cycles of 45 s at 94°C, followed by 1 min at 50°C, then 1 min 30 s at 72°C; and finally 5 min at 72°C. The amplicon was purified and used for preparation of a library by means of the MiSeq Reagent Kit v2 nano (Illumina, San Diego, CA, USA) for sequencing on Illumina MiSeq. The amplicon library concentrations were determined using BioAnalyzer DNA 1000 (Agilent Technologies, Santa Clara, CA, USA). Quality of the sequencing analysis was verified by examining a PhiX library prepared from a PhiX spike-in

control (Illumina). Sequence reads with a low quality score (Phred quality score ≤ 30) were eliminated using the fastx_trimmer tool, and then paired-end sequence reads were assembled in the paired-end assembler for the Illumina sequence software package (PANDAseq) (Masella et al., 2012). Nucleic acid sequences with ≥97% of similarity were grouped into an operational taxonomic unit (OTU) by the UCLUST algorithm (Edgar, 2010).

Phylogenetic affiliations of the OTUs were identified using a blastn search against reference sequences in the Greengenes database, version 13_5 (DeSantis et al., 2006) and the nr database (National Center for Biotechnology Information). Phylogenic tree was constructed as described above, and the 16S rRNA gene of *Methanopyrus kandleri* (GenBank accession number AB301476) was used as an outgroup. Species richness estimates Chao1 and phylogenetic diversity (PD) were calculated in the Quantitative Insight into Microbial Ecology (QIIME) software, version 1.7.0. (Caporaso et al., 2010). The Simpson index-meaning species evenness-was also calculated in QIIME. Principle component analysis (PCA) and Welch's t test were performed in the STAMP software (Parks and Beiko, 2010).

5.2.6 Accession numbers

The partial 18S rRNA gene sequences of *Metopus* sp. and *Caenomorpha* sp. were deposited in the GenBank/EMBL/DDBJ databases under the accession numbers LC027270 (1,190 bp) and LC027271 (1,195 bp). The bacterial and archeal 16S rRNA gene sequences are available under accession numbers AB938329 to AB948126 and LC152435 to LC152737.

5.3 Results

5.3.1 Reactor performance

Two UASB reactors were operated in parallel with continuous feeding of domestic sewage for 171 d. Reactors operation were stopped because granular floatation occurred at Run4 (172 d) in control reactor. The coculture and control reactors showed similar COD and SS removal efficiencies (Table 5-1); i.e. average total COD and SS removal efficiency were >57% and >85%, respectively. Methanogenic activities of the coculture reactor from Run 2 to Run 4 were significantly higher ($P < 0.05$, Student's t test) than those of the control reactor. The activities increased with the increase of COD loading rates and reached 7.3 ± 0.7 and 4.7 ± 1.5 mL (g Sludge)$^{-1}$ d^{-1} (average ± standard deviation) in the coculture and control reactors, respectively, during Run 4.

5.3.2 Population dynamics of the Metopus and Caenomorpha ciliates

Abundance of ciliates in the coculture reactor increased with the increase in COD loading rates (Fig. 5-2). In the coculture reactor, two types of morphologically different ciliates were found under an optical microscope (Fig. 5-3a and 5-3b). They were swimming around the granular sludge and

Table 5-1. Performance of the coculture reactor and control reactor. Two up-flow anaerobic sludge blanket (UASB) reactors—with and without ciliates (i.e., coculture and control reactors, respectively)—were operated for 171 d. COD: chemical oxygen demand, SS: suspended solids, n.d.: not determined. Data are presented as the mean ± standard deviation.

period (d)	HRT (h)	reactor	sample (N)	COD removal total (%)	COD removal soluble (%)	SS removal (%)	methane production ml (g Sludge)$^{-1}$ d^{-1}	retained sludge (g l^{-1})
Run 1	10	coculture	N = 15	75 ± 7	52 ± 9	97 ± 2	n.d.	38.5
0 - 37		control		73 ± 7	51 ± 8	97 ± 1	n.d.	
Run 2	6	coculture	N = 19	73 ± 13	51 ± 17	97 ± 2	1.4 ± 1.1	31.5
83 - 138		control		67 ± 13	50 ± 16	95 ± 3	0.6 ± 0.5	35.2
Run 3	3-4	coculture	N = 16	64 ± 16	48 ± 11	95 ± 4	3.5 ± 1.2	31.1
139 - 159		control		61 ± 21	46 ± 12	93 ± 5	2.3 ± 0.9	33
Run 4	1-2	coculture	N = 12	67 ± 14	50 ± 6	92 ± 7	7.3 ± 0.7	27.6
160 - 171		control		57 ± 19	47 ± 10	85 ± 16	4.7 ± 1.5	31.2

predating microbial cells. On the basis of the morphological features, those protists were identified as *Metopus* sp. and *Caenomorpha* sp.; Caenomorpha ciliates accounted for 80% of the total population of ciliates. Proliferation of protists other than the *Metopus* sp. and *Caenomorpha* sp. was not found in the coculture reactor. The phylogenetic affiliations were further analyzed by determining 18S rRNA gene sequences amplified from physically isolated *Metopus* and *Caenomorpha* ciliate cells. The 18S rRNA gene sequences that were obtained from the *Metopus* and *Caenomorpha* ciliate cells were affiliated with the family Metopidae and Caenomorphidae, respectively (Fig. 5-4); sequence similarity of the 18S rRNA gene to Metopus contortus was 95% (accession number Z29516) and to an uncultured *Caenomorpha*-like ciliate 97% (AY821933), respectively. The *Caenomorpha* ciliates contained

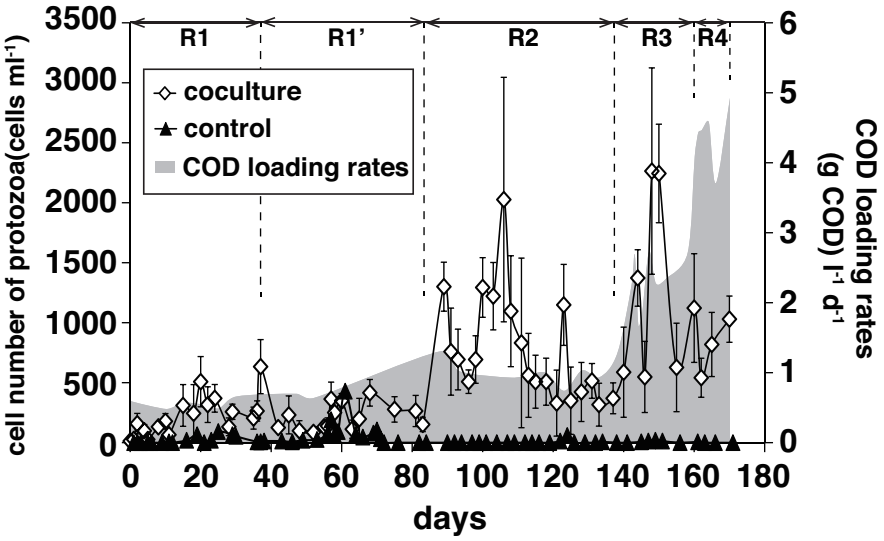

Figure 5-2. Abundance of *Metopus* and *Caenomorpha* ciliates and total chemical oxygen demand (COD) loading rates in up-flow anaerobic sludge blanket (UASB) reactors: R1, Run 1; R1', Run 1'; R2, Run 2; R3, Run3; and R4, Run4. Error bars represent standard deviation.

endosymbiotic methanogens (i.e., *Methanobacterium* sp.) according to F420-fluorescence (Fig. S5-1) and 16S rRNA gene amplicon sequencing data (Table S5-2) (van Bruggen et al., 1983); this finding is in agreement with the other studies, which also showed the presence of endosymbiotic methanogens in *Caenomorpha* ciliates (Finlay et al., 1991).

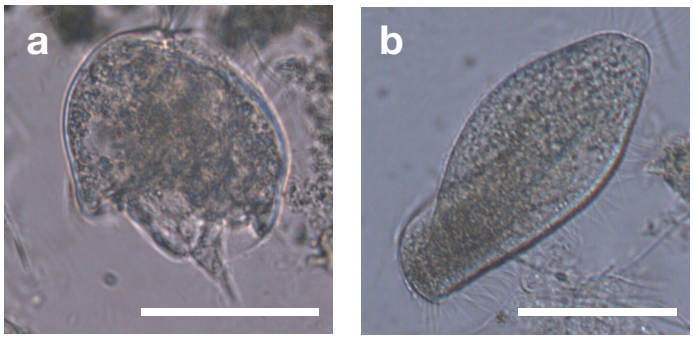

Figure 5-3. Microscopic observation of ciliates. (a) and (b) Microscopy of the *Metopus* and *Caenomorpha* ciliates that were found in the UASB reactor. The scale bar is 50 μm.

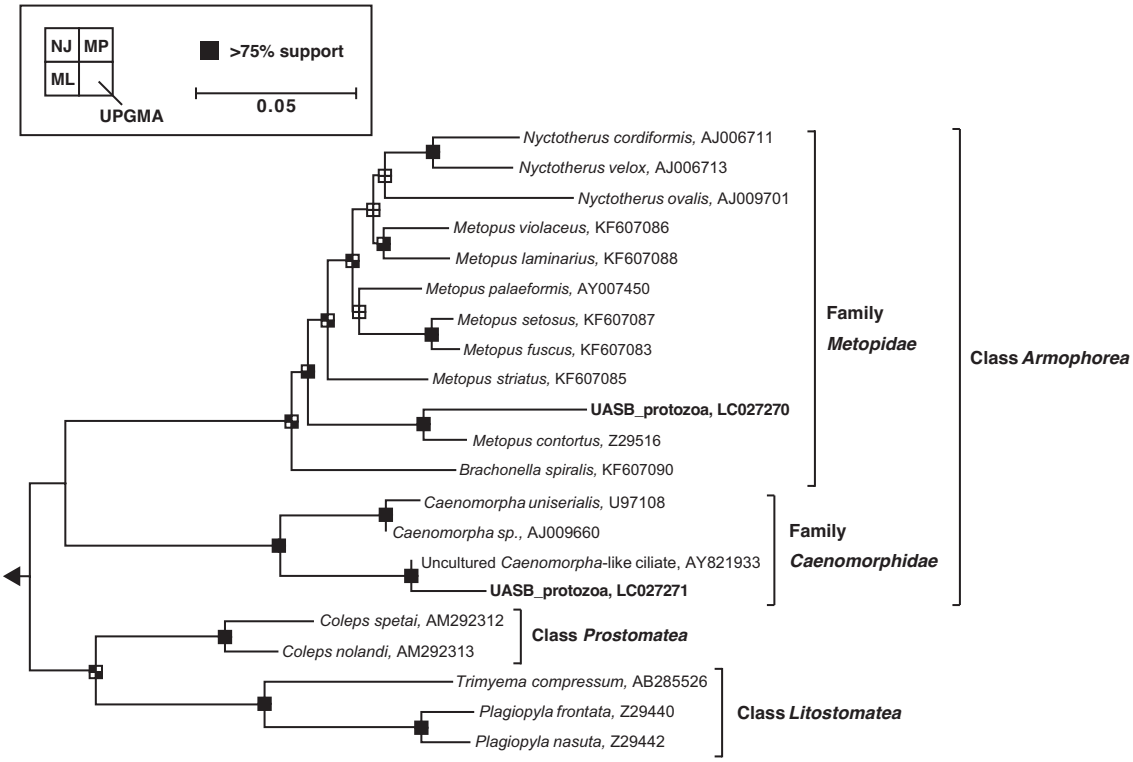

Figure 5-4. 18S-rRNA based neighbor joining (NJ) tree showing phylogenetic affiliation of the *Metopus* and *Caenomorpha* ciliates found in an up-flow anaerobic sludge blanket (UASB) reactor. Branching points that support probability >75% in the bootstrap analyses (based on 1000 replicates, estimated using the NJ method, maximum likelihood method [ML], maximum parsimony method [MP], and unweighted pair group method with an arithmetic mean [UPGMA]) are shown as black squares. The scale bar represents 5% sequence divergence.

5.3.3 Structure of the bacterial and archaeal communities

These structures in the seeding anaerobic granular sludge (0 d) and the sludge collected from the coculture and control reactors after 171 d of reactor operation were examined by sequencing PCR-amplified 16S rRNA gene regions using Illumina MiSeq. Two replicate libraries were prepared from each sample, and a total of 14,367 to 32,809 valid bacterial and archaeal sequences were recovered (Table 5-2). The sequences were clustered into OTUs (≥97% sequence similarity), and community structures in each sludge samples were compared by PCA (Fig. S5-2). The PCA analysis indicated that shifts in community structure occurred during the 171 d of reactor operation. A number of OTUs increased from the 0 d to 171 d of reactor operation, and a larger number of OTUs was found in the coculture reactor than in the control reactor: 2,885–3,190 and 2,387–2,426 OTUs, respectively (Table 5-2). The values of the species richness estimates Chao1 and PD were also greater in the coculture reactor, indicating that the bacterial and archaeal community structures in the coculture reactor were more diverse than those in the control reactor.

Table 5-2. Community richness, diversity, and evenness indices of anaerobic granular sludge collected from the coculture reactor and control reactor. Anaerobic granular sludge was collected after 171 d of operation, and two replicate libraries were prepared for each sludge sample. OTU: operational taxonomic unit (≥97% sequence similarity), PD: phylogenetic diversity.

	Number of sequence reads	OTUs	coverage	Chao1	PD	Simpson
seed sludge	14,367	1,600	0.92	8,914	115	0.91
(N=2)	14,422	1,436	0.93	7,526	107	0.91
coculture	32,809	3,134	0.95	9,498	204	0.99
(N=2)	27,870	2,839	0.94	8,127	191	0.99
control	28,277	2,372	0.96	5,052	162	0.99
(N=2)	30,378	2,321	0.96	4,717	158	0.99

Bacteria accounted for 96% of the total number of sequences, and taxonomic classification of the bacterial communities was shown in Fig. 5-5a. The class *Deltaproteobacteria* and the phyla *Bacteroidetes* and *Firmicutes* dominated both systems, while relative abundance was different between the samples; i.e. *Deltaproteobacteria* were more abundant but *Bacteroidetes* and *Firmicutes* were less abundant in the coculture reactor. Especially, relative abundance of the following bacterial genera was different between the control and coculture reactors: the *Syntrophus*, *Syntrophorhabdus, Syntrophobacter*, *Desulforhabdus, Desulfovirga* (class *Deltaproteobacteria*), *Paludibacter*, OTU-Blvii28 (phylum *Bacteroidetes*), and *Clostridium* (phylum *Firmicutes*) (Fig. 5-5b).

Taxonomic classification of the archaeal community structures was shown in Fig. 5-6a. All the sequence reads were affiliated with the archaeal classes *Methanobacteria*, *Methanomicrobia*, or

Thermoplasmata in the phylum *Euryarchaeota.* The class *Methanobacteria* and *Methanomicrobia* were abundant in both systems, while the class *Methanobacteria* was more abundant in the coculture reactor than in the control reactor. Phylogenetic analysis of archaeal 16S rRNA gene sequeneces indicated that the genus *Methanobacterium* were more abundant while *Methanosaeta* were less in the coculture reactor (Fig. 5-6b).

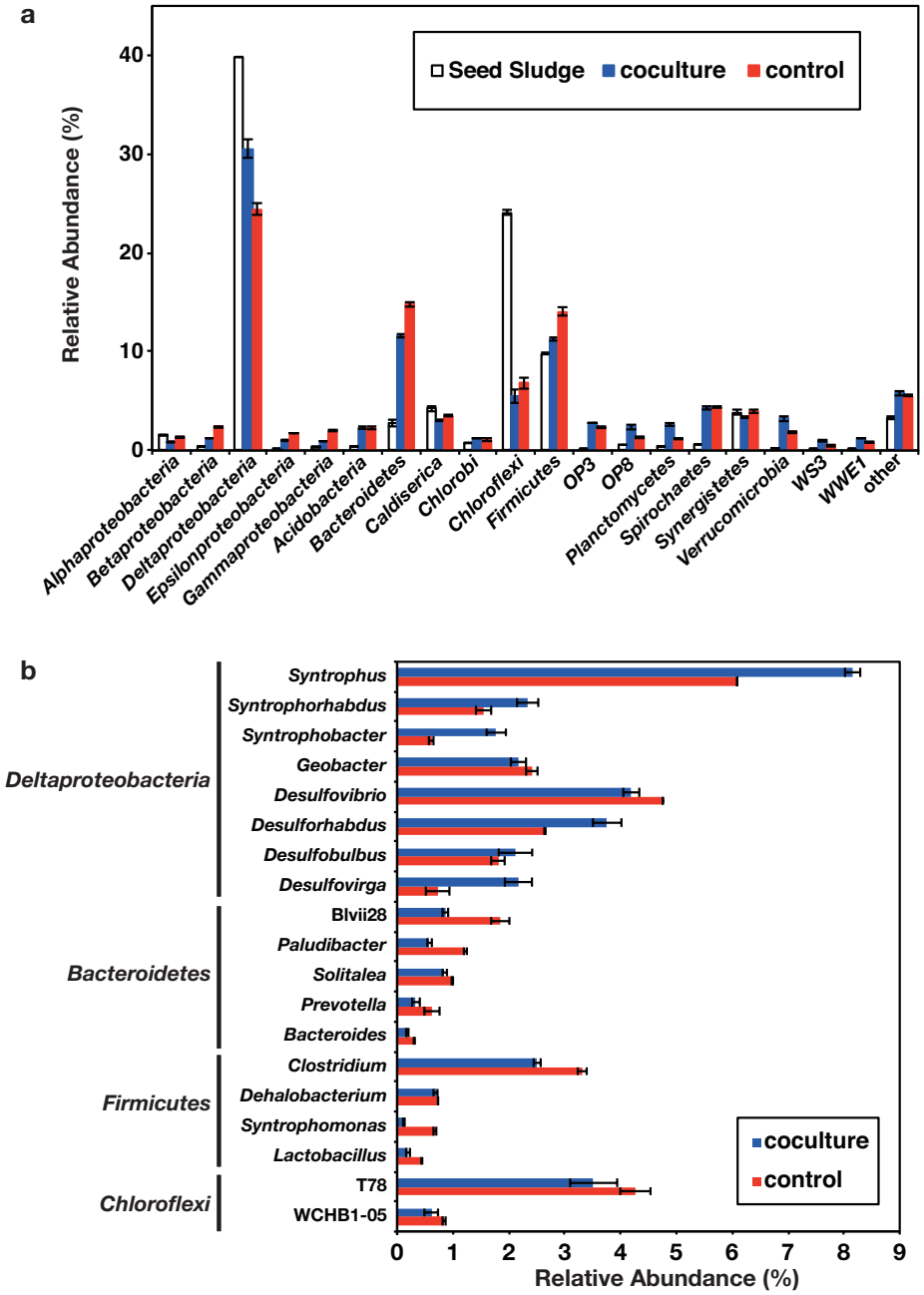

Figure 5-5. Taxonomic classification of the bacterial communities in anaerobic granular sludge collected after 171 d of operation of an up-flow anaerobic sludge blanket (UASB) reactor. (a) Abundance of 16S rRNA gene sequences from each bacterial phylum and class. Sequence reads that are not classified into any known bacterial group were labeled as "other". Approximately 4% of all reads were archeal 16S rRNA gene sequences, which are not shown in this figure (but shown in Fig. 3-6a). (b) Abundance of 16S rRNA gene sequences from each bacterial genus in major bacterial group (the class *Deltaprteobacteria* and the phylum *Bacteroidetes*, *Firmicutes* and *Chloroflexi*). Coculture and control: UASB reactors with and without *Metopus* and *Caenomorpha* ciliates, respectively.

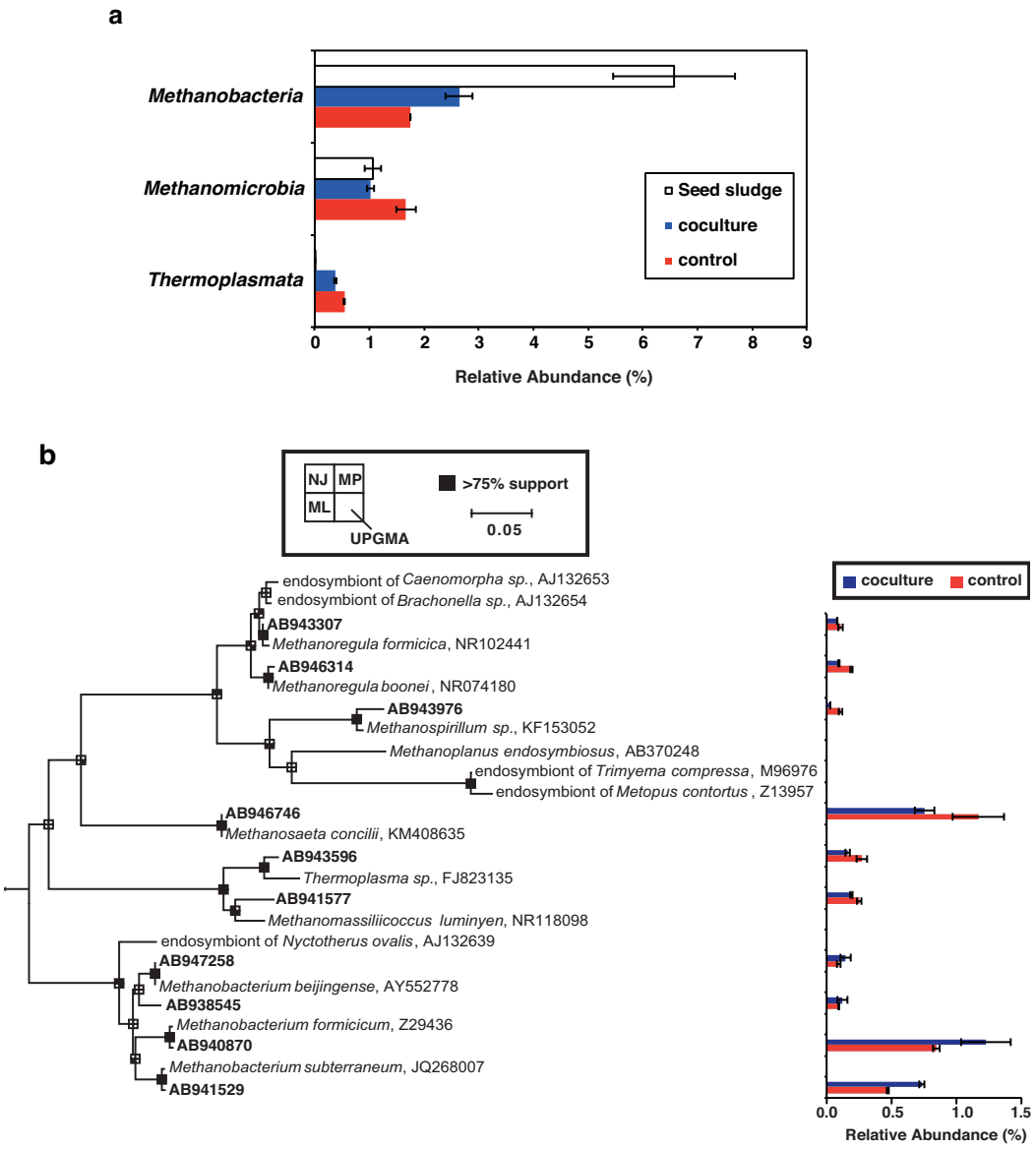

Figure 5-6. Taxonomic classification of the archaeal communities in the anaerobic granular sludge that was collected after 171 d of operation of an up-flow anaerobic sludge blanket (UASB) reactor. (a) Abundance of 16S rRNA gene sequences from each archaeal class. Coculture and control: UASB reactors with and without *Metopus* and *Caenomorpha* ciliates, respectively. (b) 16S rRNA-based neighbor-joining (NJ) tree showing phylogenetic affiliations of archaeal operational taxonomic units (OTUs) in the UASB reactors. Boldface indicates OTUs found in the present study. The scale bar represents substitution of 5% of bases. Branching points that support probability >75% in the bootstrap analyses (based on 1,000 replicates, estimated using the NJ method, maximum likelihood method [ML], maximum parsimony method [MP], and unweighted pair group method with an arithmetic mean [UPGMA]) are shown as black squares. Abundance of sequence reads that are affiliated with each OTU is shown in the graph on the right.

5.4 Discussion

Cocultivation of ciliates and prokaryotic assemblages is a first step during analysis of the impact of predation by protists, and long-term cocultivation is necessary to assess this impact because the rates of consumption of prokaryotic standing stocks by ciliates are generally low in anoxic ecosystems (Finlay et al., 1991; Massana and Pedrós-Alió, 1994; Saccà et al., 2009). *Metopus* ciliates were previously detected in a UASB reactor processing diluted wastewater (Agrawal et al., 1997); this finding is suggestive of suitability of a UASB reactor as a cultivation tool for those ciliates. In the present study, it was demonstrated that a UASB reactor fed with sewage allows for cocultivation of *Metopus* and *Caenomorpha* ciliates and prokaryotic assemblages for 171 d. Notably, *Caenomorpha* ciliates have been detected in a wide range of natural freshwater systems (Finlay et al., 1991; Guhl and Finlay, 1993; Massana and Pedrós-Alió, 1994), whereas cultivation of the *Caenomorpha* ciliates has been rarely described to date. UASB reactors have excellent capacity for biomass retention (Lettinga et al., 1980); this characteristic probably allows for proliferation of the slow-growing *Metopus* and *Caenomorpha* ciliates; the doubling time of these ciliates in the coculture reactor were roughly estimated to be 2.5–5 d, judging by the increase in their cell numbers after 96–106 d and 137–148 d (Fig. 5-2). In addition, sulfide-rich sewage (4.3 mg-S L-1 i.e. 0.13 mM; Table S5-1) was fed into the UASB reactors; this situation possibly resulted in the selective growth of *Metopus* and *Caenomorpha* ciliates. These ciliates prefer sulfide-rich (>1 mM) ecosystems (van Bruggen et al., 1986; Finlay et al., 1991; Massana et al., 1994), whereas other anaerobic ciliates are sensitive to sulfide (Massana and Pedrós-Alió, 1994); e.g. the minimum inhibitory concentration of sulfides for *Coleps* ciliates is 0.01 mM (Pedrós-Alió et al., 1995). In the present study, *Metopus* and *Caenomorpha* ciliates could be cultivated in the range of 10^2 to 10^3 cells mL^{-1}, which is higher than that in a freshwater lake ($<10^2$ cells mL^{-1}) but smaller than that in the rumen (10^5 cells mL^{-1}) (Dehority, 1984; Guhl et al., 1996; Massana and Pedrós-Alió, 1994).

Methane production was more prominent in the coculture reactor; this result is consistent with earlier observations (Biagini et al., 1998; Holmes et al., 2014). In those studies, the increase in specific microbial activities of methanogenesis and sulfate reduction was found in anoxic ecosystems with protists. Although *Caenomorpha* ciliates contain endosymbiotic methanogens (Fig. S5-1), the contribution of symbionts to the increase in methanogenic activities is expected to be minor. If the abundance of the ciliates and endosymbiotic methanogens and specific methanogenic activities by the symbionts are assumed to be 2,500 ciliates $\cdot$ ml^{-1} (Fig. 5-2), 4,500 cells $\cdot$ $ciliates^{-1}$ (van Bruggen et al., 1984; Schwarz and Frenzel, 2005), and 0.97 (fmol methane) $\cdot$ $endosymbiont^{-1} \cdot h^{-1}$ (Finlay et al., 1991), respectively, then the contribution to the methane production during Run 3 is estimated to be less than 0.1%. Instead of the symbiotic contribution, the *Metopus* and *Caenomorpha* ciliates possibly stimulated microbial activities in our experiments, through decomposition of organic material and processing of minerals as demonstrated previously on aerobic protists (Fenchel and Harrison, 1976; Bloem et al.,

1988). It should be noted that total COD and SS removal efficiencies were similar between the coculture and control reactors while methane gas production was greater in the coculture reactor (Table 5-1). The greater methane gas production in the coculture reactor possibly resulted from degradation of particular COD fractions to soluble organic matters by the ciliates (Narayanan et al., 2007). Additionally, hydrogenotrophic methanogens (i.e. *Methanobacterium*) were more abundant while acetotrophic methanogen (i.e. *Methanosaeta*) were less in the coculture reactor (Fig. 5-6b). Greater abundance of hydrogenotrophic methanogens in the coculture reactor possibly contributed to increase of methanogenic activities in coculture reactor because hydrogenotrophic methanogens showed higher methanogenic activities than those of acetotrophic methanogen (Tagawa et al., 2000). Methane gas production and its recovery in the UASB reactors have been implemented for the use of methane gas as an energy source alternative to fossil fuels. For this purpose, an increase in methanogenic activities is desirable, and the mechanisms involved should be explored in another study.

Sequencing of 16S rRNA gene amplicons using Illumina MiSeq was carried out to investigate the influence of predation by *Metopus* and *Caenomorpha* ciliates to prokaryotic community structure and diversity. The community structure and diversity were investigated in the seeding anaerobic granular sludge and the sludge collected from the coculture and control reactors after 171 d of reactor operation. Our outcomes showed that predation by Metopus and Caenomorpha ciliates resulted in alteration of microbial community structure and diversity. This finding was consistent with the previous studies describing that predation by aerobic ciliates enhanced community evenness and diversity (Bell et al., 2010; Saleem et al., 2012). Although grazing by the *Metopus* and *Caenomorpha* ciliates on prokaryotic assemblages was not measured directly in this study, all of the ciliates contained food vacuoles packed with prokaryotic cells as determined by optical microscopy, strongly suggesting that the ciliates were consuming the prokaryotic cells. Indeed, the amounts of microbial biomass retained in a UASB reactor were smaller in the coculture reactor than in the control reactor (Table 5-1); this phenomenon probably resulted from the predation by the protists.

Abundance of 16S rRNA gene sequences of *Paludibacter*, OTU-Blvii28, *Clostridium* was lower in the coculture reactor; this effect may have resulted from selective grazing by the *Metopus* and *Caenomorpha* ciliates. These ciliates were swimming around prokaryotic assemblages (i.e. granular sludge) and consuming microbial cells; therefore, the microbial cells located in the outer layer of the granular sludge were preferentially eaten by the ciliates. The outer layer of the granular sludge in the UASB reactor was dominated by bacterial cells and performed the function of initial anaerobic degradation of complex organic compounds to simpler ones (Sekiguchi et al., 1999; Fang, 2000). Bacteria affiliated with the genera *Paludibacter*, OTU-Blvii28 (Bacteroidetes) (Ueki et al., 2006; Su et al., 2014), and *Clostridium* (Firmicutes) (Chen et al., 2005; Lo et al., 2010) were rod- or coccus-shaped bacteria producing extracellular hydrolase and contributed to the anaerobic degradation. Those bacteria

were previously found in the outer layer of granular sludge in a UASB reactor, as determined by fluorescence in situ hybridization analysis (Fang, 2000; Liu et al., 2002, Tay et al., 2002). On the other hand, filamentous bacteria affiliated with the phylum Chloroflexi were also previously found in the outer later of granular sludge in a UASB reactor. In the present study, 16S rRNA gene sequences of OTU-T78 and OTU-WCHB1-05 showed 91% and 92% of sequence similarities to the 16S rRNA gene sequence of *Leptolinea tardivitalis* (accession number; NR_040971), a known filamentous bacterium affiliated with the phylum Chloroflexi (Yamada et al., 2005; 2006; 2009). As shown in Fig. 5-5b, abundance of 16S rRNA sequences affiliated with the OTU-T78 and OTU-WCHB1-05 were not different between the coculture and control reactors. This finding suggested that *Metopus* and *Caenomorpha* ciliates selectively grazed the rod or coccus-shaped bacterial cells located in the outer layer. These predation behaviors of these ciliates are in consistent with the results of feeding experiment of *Cyclidium* ciliate in Chapter IV.

In Chapter IV, ingestion rates of protists seemed to logarithmically proportional to the cell volume to the power of about 0.75. Thus, ingestion rate might be roughly estimated based on protist cell size to the power of 0.75. The cell volumes of protist *Metopus* and *Caenomorpha* were approximately 1.6×10^5 and 9.2×10^4 μm^3, respectively. The ingestion rates of ciliates *Metopus* and *Caenomorpha* were estimated to 1.1×10^5 and 7.4×10^4 cells of bacteria protist^{-1} hour^{-1} from their cell volume and our data of *Cycliidum* sp. YH. In UASB reactor, number of ciliates were ranged 10^2-10^3 cells mL^{-1} (Fig. 5-2) and combining the estimated ingestion rate, it is assumed that the maximum contribution of ciliates for control of bacterial populations is estimated up to 1.8×10^9 cells of bacteria mL^{-1} day^{-1}. Although this contribution during Run 3 is only 0.2% day^{-1} of prokaryotes (both bacteria and archaea) of granular sludge (more than 10^{12} cells mL^{-1}), it is increased 35.7% day^{-1} if limited to bacteria located in the outer layer of granular sludge that could be ingested by protist (ca. 5×10^9 cells mL^{-1}) (Wu et al., 1992). Therefore, predation by these ciliates could strongly influence on microbial community structures, although detailed ingestion rate of ciliates *Metopus* and *Caenomorpha* needs to examine in future study.

The 16S rRNA gene sequences of *Syntrophus*, *Desulfovirga*, *Syntrophobacter*, and *Syntrophorhabdus* were more abundant in the coculture reactor (Fig. 5-5b). Those bacteria prefer volatile fatty acids as a carbon source (Boone and Bryant, 1980; Mountfort et al., 1984; Tanaka et al., 2000; Qiu et al., 2008), whereas the concentrations of these fatty acids were always below the detection limit (<0.1 mM) in both UASB reactors in our study. *Metopus* (van Bruggen et al., 1986; Esteban et al., 1994) and *Caenomorpha* (Finlay, 1981) ciliates have a unique organelle, hydrogenosome, instead of mitochondria, in which organic matter is oxidized to volatile fatty acids (i.e. acetate, valeric acid, and lactic acid) and hydrogen for ATP synthesis (Yarlett et al., 1985; Goosen et al., 1990; Müller, 1993). It is possible that the *Metopus* and *Caenomorpha* ciliates produced volatile fatty acids in the

hydrogenosome, and this situation ensured greater proliferation of Syntrophus, *Desulfovirga*, *Syntrophobacter*, and *Syntrophorhabdus* in the coculture reactor. Additionally, the hydrogenosome of *Metopus* and *Caenomorpha* ciliates produces hydrogen, which enabled greater proliferation of hydrogenotrophic methanogens (i.e. *Methanobacterium*) in the coculture reactor (Fig. 5-6b) as demonstrated previously (Müller, 1993; van Hoek et al., 2000; Hirakata et al., 2015). Almost nothing is known about the underlying symbiotic associations between the ciliates and prokaryotic assemblages in UASB reactors, and specific interactions between the ciliates and *Syntrophus*, *Desulfovirga*, *Syntrophobacter*, *Syntrophorhabdus*, and *Methanobacterium* must be examined in future studies.

Predation by *Metopus* and *Caenomorpha* ciliates altered prokaryotic community structure, diversity, and functioning (i.e. methanogenesis) in our UASB reactors. *Metopus* and *Caenomorpha* ciliates have been found in a wide range of anoxic ecosystems including anoxic freshwater (Massana and Pedrós-Alió, 1994; Bourland et al., 2014), marine sediments (Esteban et al., 1994; van Bruggen et al., 1986), landfill sites (Fenchel and Finlay., 1990; Finlay and Fenchel, 1991), and a rice field soil (Schwarz and Frenzel, 2005). Predation by protists needs to be taken into consideration in the future for a better understanding of prokaryotic ecology in such anoxic ecosystems.

5.5 Summary of this chapter

In this study, we investigate the influence of predation by anaerobic protists such as *Metopus* and *Caenomorpha* ciliates on prokaryotic community function, structure, and diversity. *Metopus* and *Caenomorpha* ciliates were cocultivated with prokaryotic assemblages in lab-scale UASB reactor for 171 d. Predation by these ciliates increased the methanogenic activities of granular sludge, which constituted 155% of those found in a UASB reactor without ciliates. Sequencing of 16S rRNA gene amplicons revealed that the prokaryotic community in the UASB reactor with the ciliates was more diverse than that in the reactor without ciliates; 2,885–3,190 and 2,387–2,426 OTUs, respectively. The effects of predation by protists in anaerobic engineered systems have mostly been overlooked, and our results show that the influence of predation by protists needs to be examined and considered in the future for a better understanding of prokaryotic community structure and function.

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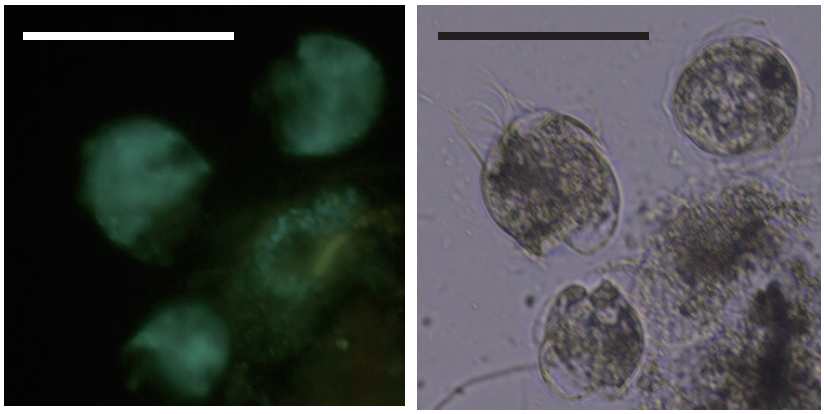
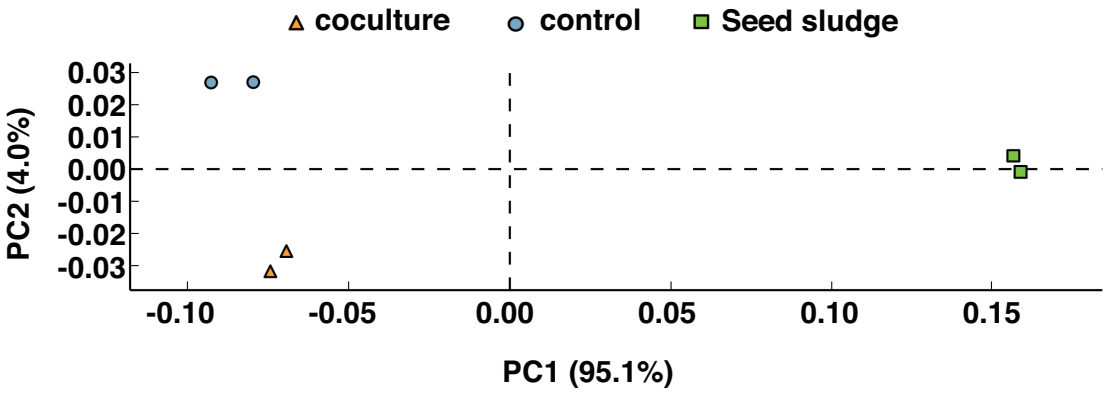
5.7 Supplementary Information



Supplementary Figure S5-1. Endosymbiotic methanogens in *Caenomorpha* ciliates. Left: F_{420} fluorescence, right: a phase contrast image. The scale bar is 100 μm.



Supplementary Figure S5-2. Comparison of the prokaryotic community structures found in seeding anaerobic granular sludge (square) and the sludge collected from coculture and control reactors after 171 d of operation (triangle and circle, respectively). Principle component analysis (PCA) of community structures at operational taxonomic units (OTU) (≥97% sequence similarity) level.

Supplementary Table S5-1. Composition of the sewage that was fed into up-flow anaerobic sludge blanket (UASB) reactors in the present study. CODCr: chemical oxygen demand determined using a dichromate, BOD5: biological oxygen demand for 5 d, SS: suspended solids, VSS: volatile suspended solids, TKN: total Kjeldahl nitrogen, T-N: total nitrogen, and T-P: total phosphorus. The soluble fraction was prepared by filtration through the glass fiber membrane GB-140. The data are presented as mean ± standard deviation.

COD_{Cr} (mg L^{-1})		BOD_5 (mg L^{-1})		SS (mg L^{-1})	VSS (mg L^{-1})	Sulfide (mg S) L^{-1}
Total	Soluble	Total	Soluble			
269 ± 73	122 ± 34	182 ± 92	87 ± 37	80 ± 37	71 ± 27	4.3 ± 0.7

SO42- (mg S) L^{-1}	NH4+ (mg N) L^{-1}	NO3- (mg N) L^{-1}	NO2- (mg N) L^{-1}	TKN (mg N) L^{-1}	T-N (mg N) L^{-1}	T-P (mg P) L^{-1}
14 ± 17	21 ± 6	0.3 ± 0.05	0.18 ± 0.17	29 ± 5	29 ± 5	3.5 ± 0.7

Supplementary Table S5-2. Taxonomic classification of the prokaryotic communities in Caenomorpha ciliate cell. A single Caenomorpha ciliate cell was separated after 171 d of operation using a micromanipulator, and the prokaryotic 16S rRNA gene sequence was amplified by PCR using oligonucleotide primers 515F and 806R as previously described (Hirakata et al., 2015). The amplified 16S rRNA gene sequence was determined using the MiSeq sequencer (Illumina, San Diego, CA, USA), and the sequence data were analyzed using the QIIME software (version 1.8.0). OTU: operational taxonomic unit (≥97% sequence similarity). OTUs that abundance was less than 1% were grouped and labeled as "other". n.a: not applicable.

OTU	Accession number	Relative abundance	Closely related species (accession number)	Domain	Sequence similarity (%)
denovo1	LC152435	89.7%	*Methanobacterium* sp. (KJ432636.1)	Archaea	100%
denovo2	LC152436	3.6%	*Lactobacillus sakei* (KT968365.1)	Bacteria	100%
denovo3	LC152437	1.7%	*Escherichia coli* (KU161315.1)	Bacteria	100%
other	*n.a*	5.0%	*n.a*	*n.a*	*n.a*

Reference

Hirakata, Y., M. Oshiki, K. Kuroda, M. Hatamoto, K. Kubota, T. Yamaguchi, H. Harada and N. Araki. 2015. Identification and detection of prokaryotic symbionts in the ciliate *Metopus* from anaerobic granular sludge. Microbes Environ. 30: 335-338.

Chapter VI: Summary of this thesis

6.1 Summary

This thesis focused on anaerobic protist in the UASB reactor. Little is known about protist function and community in anaerobic wastewater treatment systems because most of anaerobic protist species are still largely uncultured or overlooked. Therefore, for clarify protist community and function in anaerobic wastewater treatment processes, molecular biological method, batch experiment, and long-term continuous cultivation by lab-scale UASB reactor were performed. The experimental outcomes and conclusions in each chapter are as follows:

In Chapter III, protist community structures in a UASB reactor treating domestic sewage were characterized by the 18S rRNA gene amplicon sequencing. Many protist groups that are barely observable microscopically could detect by 18S rRNA gene amplicon sequencing. These protist phyla that overlooked by microscopic observation in V4 and V9 amplicon libraries were accounted with 72.8% and 89.0% of total protist sequences, respectively. As the results of V4 amplicon libraries that specifically amplified eukaryotic sequences, phylum Ciliophora was most dominant throughput the years in the UASB reactor. CCA analysis indicated that protist genera *Cyclidium*, *Platyophrya* (phylum Ciliophora) and *Subulatomonas* (phylum Sulcozoa) correlated with chemical oxygen demand and suspended solid concentration, and could be used as bio-indicators of treatment performance.

Chapter IV described establishement of monoxenic culture of three anaerobic protists, *Cyclidium* sp. YH (phylum Ciliophora), *Paracercomonas* sp. YH (phylum Cercozoa), and *Trichomitus* sp. YH (phylum Metamonada), isolated from UASB reactor treating domestic sewage. Tracer experiments using GFP and stable carbon isotope showed that these three protists could ingest and digest bacteria cell. In addition, tracer experiments using stable carbon isotope demonstrated that *Cyclidium* sp. YH directly supplied CO_2 and hydrogen to endosymbiotic methanogen. Thus, anaerobic protists harbor endosymbiotic methanogen could contribute to methane production. The ingestion rates of *Cyclidium* sp. YH, *Paracercomonas* sp. YH, and *Trichomitus* sp. YH were 1.5-2.7 $\times$ 10^3, 1.3-1.5 $\times$ 10^2 and 0.4-0.6 $\times$ 10^2 cells·protist^{-1}·hour^{-1}, respectively. Our data and previous study indicated that ingestion rates of protist seem to logarithmically proportional to the cell volume regardless of either anaerobic or aerobic species. Ingestion rates of anaerobic protists would be important information for consideration of their contributions to sludge reduction in anaerobic ecosystems. In particular, ingestion rate of *Cyclidium* sp. YH were the fastest of three protists, suggesting that *Cyclidium* sp. YH may contribute sludge reduction and treatment performance. Importance of *Cyclidium* sp. in the UASB reactor treating domestic sewage was suggested in also Chapter III. *Cyclidium* sp. YH and *Trichomitus* sp. YH could ingest both gram negative- and positive- bacteria although *Paracercomonas* sp. could ingest only gram-negative bacteria. Notably, only *Trichomitus* sp. YH could ingest filamentous bacteria (*T. flocculiformis*). All protists in this study can not grow when archaea was used as substrate. Feeding

experiments showed that food bacteria species influenced generation time, metabolite of anaerobic protists. These difference of predation behaviors suggested that three protists may play different roles and have various effects on microbial community structures in anaerobic ecosystems.

In Chapter V, the influence of predation by anaerobic protist such as *Metopus* and *Caenomorpha* ciliates on prokaryotic community function, structure, and diversity in the UASB reactor was investigated. *Metopus* and *Caenomorpha* ciliates were cocultivated with anaerobic granular sludge in UASB reactor for 171 day. Predation by these ciliates increased the methanogenic activities of granular sludge, which constituted 155% of those found in a UASB reactor without the ciliates. Sequencing of 16S rRNA gene amplicons revealed that the prokaryotic community in the UASB reactor with the ciliates was more diverse than that in the reactor without ciliates. In addition, syntrophic bacteria (i.e., *Syntrophus*, *Desulfovirga*, *Syntrophobacter*, and *Syntrophorhabdus*) were more abundant in the coculture reactor, while abundance of fermentative bacteria such as *Paludibacter*, OTU-Blvii28, *Clostridium* were lower in the UASB reactor with the ciliates. This effect may have resulted from selective grazing by the *Metopus* and *Caenomorpha* ciliates because ciliates were swimming around granular sludge and consuming fermentative bacteria cells that located outer layer of granule (Figure 6-1). Growth of syntrophic bacteria suggested that the *Metopus* and *Caenomorpha* ciliates may have supplied hydrogen and volatile fatty acids to ecosystems (Figure 6-1). Moreover, these effects by protist may also result in stimulation of methanogenic activities in anaerobic ecosystem.

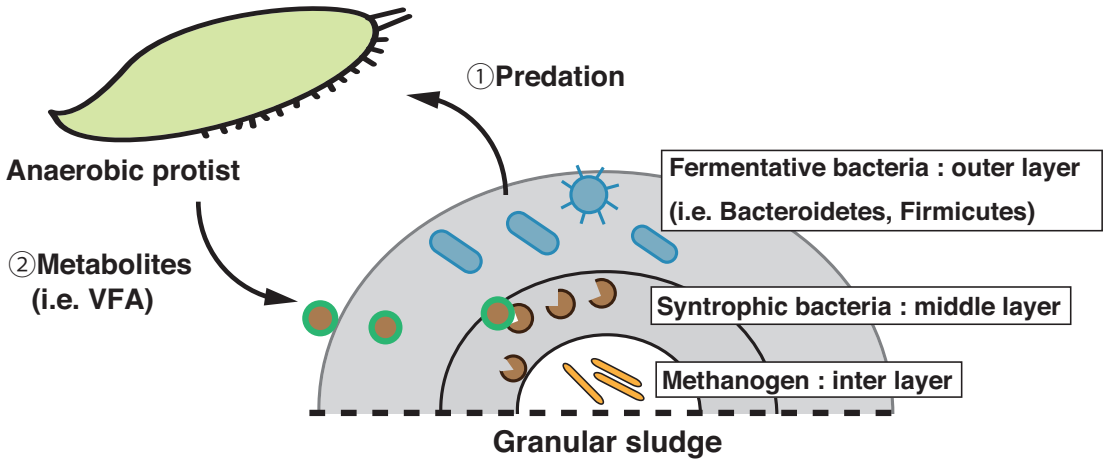


Figure 6-1. Hypothetical scheme role of anaerobic protist in anaerobic granular sludge.

Over all, this study presented in this thesis revealed involvement of anaerobic protist in treatment performance and prokaryotic community of UASB reactor treating domestic sewage. In addition, these results showed anaerobic protist relevant to treatment performance, and obtained successfully monoxenic culture of three protists that have different physiological characteristics. These results

provided valuable information on anaerobic protist species involved in wastewater treatment processes and their ecological roles in anaerobic ecosystems of UASB reactor treating domestic sewage.

6.2 Future tasks

In Chapter III, the 18S rRNA gene sequenceing analysis of UASB reactor throughout two year suggested that protist genera *Cyclidium*, *Platyophrya* (phylum Ciliophora) and *Subulatomonas* (phylum Sulcozoa) could be used as bio-indicators of treatment performance in the UASB reactor treating domestic sewage. Although only genus *Cyclidium* was isolated and cultured, and its morphological and physiological information were also obtained in experiments of Chapter IV, genera *Platyophrya* and *Subulatomonas* were not observed microscopically in this study. Therefore, in order to use each protists as bio-indicators, it is necessary to link the morphological information and genetic information of these protist species in the future research. In addition, uncultured eukaryotes such as parasitic protists (phylum Apicomplexa, Ichthyosporea, and Perkinsozoa) and LKM11 and LKM15 groups of fungi (phylum Cryptomycota) were dominantly detected in the UASB reactor. The physiological roles of these eukaryotes need to be examined to understand their contributions to anaerobic processes in future studies.

In Chapter V, our results show that the influence of predation by protists needs to be examined and considered in the future for a better understanding of prokaryotic community structure and function. On the other hands, the coculture and control reactors showed similar COD and SS removal efficiencies. Although long-term cultivation experiments using lab-scale UASB reactor revealed effect of predation by protists on prokaryotic community, contribution of anaerobic protists to treatment processes was unclear. The results of Chapter IV and V indicated that more cell number of protists could have more contribution to treatment processes. Therefore, maintaining high cell density of anaerobic protists was expected to need in order to improvement of treatment performance of UASB systems by using function of protists. Moreover, effect of fungi was also unclear in this study even though they were detected by 18S rRNA gene sequencing in Chapter III. A cycloheximide inhibits growth of both protist and fungi, thus prokaryotic community might be influenced by also fungi. The ecosystems including bacteria, archaea, protist, and fungi should to be investigated for better understanding wastewater treatment processes in UASB reactor.

As mentioned above, although this thesis needs further study, we expect that follow-up studies using these data and cultures of protists obtained in this study may contribute to stabilization of domestic sewage treatment by UASB reactor in the future.

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