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ORIGINAL ARTICLE

Bacteriophage predation regulates microbial abundance and diversity in a full-scale bioreactor treating industrial wastewater

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Changes in the microbial community composition of a full-scale membrane bioreactor treating industrial wastewater were studied over a period of 462 days using a series of 16S rRNA gene clone libraries. Frequent changes in the relative abundance of specific taxonomic groups were observed. which could not be explained by changes in the reactor's conditions or wastewater composition. Phage activity was proposed to drive some of the observed changes. Bacterial hosts were isolated from a biomass sample obtained towards the end of the study period, and specific phage counts were carried out for some of the isolated hosts using stored frozen biomass samples as the phage inocula. Plaque-forming unit concentrations were shown to change frequently over the study period, in correlation with changes in the relative abundance of taxonomic groups closely related by 16S rRNA gene sequence to the isolated strains. Quantitative PCR was used to verify changes in the abundance of a taxonomic group closely related to one of the isolated hosts, showing good agreement with the changes in relative abundance in the clone libraries of that group. The emerging pattern was consistent with the 'killing the winner' hypothesis, although alternative interaction mechanisms could not be ruled out. This is the first time that phage-host interactions in a complex microbial community are demonstrated over an extended period, and possibly the first in situ demonstration of 'killing the winner' stochastic behavior.

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Introduction

The composition of a microbial community is affected by many environmental and biological factors. In recent years, there has been an increasing speculation as to the role of specific predation by bacteriophage on microbial diversity and population dynamics (Bouvier and del Giorgio, 2007). Most of the research in this field was carried out in aquatic, mainly oceanic habitats (Suttle, 2007; Zhang *et al.*, 2007; Winget and Wommack, 2008) wherein it is estimated that viral activity daily destroys over 25% of microbial cells in the oceans, with implications

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on microbial food webs, carbon turnover and global carbon cycle (Suttle, 2007). Fresh water systems have also been investigated (Middlboe et al., 2008), although less frequently. The commonly accepted paradigm for viral-bacterial interactions is that suggested by Thingstad and Lignell (1997), termed 'phage kills the winner'. This hypothesis is mainly justified by mathematical models (Thingstad, 2000; Middelboe et al., 2001; Middlboe et al., 2008; Miki et al., 2008), with some support coming from data from different aquatic environments (Zhang et al., 2007). 'Phage kills the winner' appears to be valid for simple ecosystems in laboratory experiments (Middlboe et al., 2008), but has not been directly demonstrated for specific phage-host systems active within a complex chaotic environment.

Wastewater treatment bioreactors harbor a unique type of microbial community cultivated to produce a desired effect, that is, to remove organic carbon and

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nutrients from wastewater before their release into the environment. Many efforts have been made towards better understanding of the mechanisms controlling the microbial ecology of these systems (Amann et al., 1995, 1998; Wagner et al., 2002; Daims et al., 2006; Martín et al., 2006). However, little is known about phage activity in bioreactors, a subject considered, thus far, in only a handful of publications (Ewert and Paynter, 1980; Hantula et al., 1991; Khan et al., 2002a, b; Otawa et al., 2007; Kunin et al., 2008; Wu and Liu, 2009). Many studies have focused on the detection of viral particles as indicators of survival and release of pathogenic viruses into the environment (Lucena et al., 2004; Gino et al., 2007; Guzman et al., 2007) However, there is little or no information available regarding the impact of viral activity on microbial ecology or process performance. The dense

systems ideal hunting grounds for bacteriophage. The hypothesis that phage-host interactions affect the microbial ecology and process stability of wastewater treatment bioreactors was the basis for this study. Changes in microbial community composition were followed over the first 15 months of operation of a bioreactor treating industrial wastewater. Plaque-forming units (PFU) were enumerated for selected bacterial isolates over the same period to reveal changes in the abundance of host-specific bacteriophage. The data were used to better understand phage-host dynamics and interactions in this complex environment.

biomass in engineered bioreactors, maintained under

homogenized, relatively stable conditions, makes these

Materials and methods

System description

Biomass samples were obtained from a full-scale membrane bioreactor-treating chemical industry wastewater. The reactor had an operating volume of $20\,000\,\text{m}^3$ and a hydraulic retention time of 3.3 days. Average organic matter concentration entering the system (measured as total dissolved organic carbon) during the research period was $2374 \pm$ 548 mg l^{-1} . The membrane pore size was approximately 4 µm, although biomass caking on the membrane surface effectively retained most of the bacteria, with no visible effluent turbidity. Biomass concentration started at approximately $1000 \text{ mg} l^{-1}$ and reached values as high as $22\,000\,\mathrm{mg}\,\mathrm{l}^{-1}$. Wastewater composition was not constant over the study period, as it was influenced by varying processes operated in the plant.

Sampling

A 1-I sample was obtained directly from the bioreactor aeration basin every 1–3 weeks. Two 4 ml aliquots were stored at -80 °C within 2 h of sampling until further processing (DNA extraction or filtration for PFU counts). Interruption of sampling or loss of samples created gaps of upto 46 days in the resulting data.

The ISME Journal

Phage-host interactions in a full-scale bioreactor

Construction of clone libraries

DNA extraction-A thawed biomass sample was vortexed for 30 s, 1 ml aliquot was centrifuged (3 min, 12 000 r.c.f.) and the supernatant discarded. The pellet was treated using Ultraclean Soil DNA Isolation Kit. The pellet was first re-suspended in bead solution and then added to the bead tube for DNA extraction according to manufacture protocol. DNA concentration was measured using NanoDrop ND-1000 spectrophotometer and adjusted to 6–8 ng μ l⁻¹ by dilution in molecular grade double distilled water.

16S rRNA gene amplification

16S rRNA gene was amplified by PCR from total DNA extractions using a Mastercycler gradient thermocycler (Eppendorf, Westbury, NY, USA). Amplification was carried out using modified 16S rRNA primers for bacteria. (Forward: 8F-I (5'-GGAT CCAGACTTTGATYMTGGCTCAI), and reverse: 907R-I (5'-CCGTCAATTCMTTTGAGTTI) (Ben-Dov et al., 2006)). The primers used in the PCR amplifications were obtained from Sigma-Genosys (Rehovot, Israel). Reaction mixture included 25 µl ReddyMix (PCR Master mix containing 1.5 mM MgCl₂, 0.2 mM concentration of each deoxynucleoside triphosphate) (ABgene, Surrey, UK), 2 µl each of forward and reverse primers $(10 \,\mu mol \,ml^{-1})$, $2 \,\mu l$ analytical grade BSA (2.5 mg ml⁻¹) and 2 µl of DNA template. $MgCl_2$ was supplemented to a final concentration of 5.5 mM and double distilled water was added to bring the total volume to $50\,\mu$ l. The PCR protocol included an initial step of 4 min at 95 °C, followed by 35 cycles of the following incubation pattern: 94 °C for 40 s, 50 °C for 40 s and $72 \degree C$ for 60 s. A final step at $72 \degree C$ for 20 min concluded the reaction. PCR samples were then stored at -20 °C until further processing.

Clone library construction and sequencing-PCR products were purified by electrophoresis through a 1.0% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator. The approximately 0.9kb heterologous 16S rRNA DNA products were excised from the gel, and the DNA was purified from the gel slice using the Wizard PCR Prep kit (Promega, Madison, WI, USA). The gel-purified PCR products were cloned into the pCRII-TOPO-TA cloning vector as specified by Invitrogen (Carlsbad, CA, USA), and transformed into HIT competent cells according to manufacturer's instructions. 96 colonies for each sample were picked using sterile toothpicks and clones were sequenced by MCLAB (south San Francisco, CA, USA) using the M13F primer.

Data analysis

Sequences were first screened for chimeras using the Bellerophon server (Huber *et al.*, 2004). The classifier program at the RDP site (Cole *et al.*, 2009) was then used to assign sequences to taxonomical hierarchy. Sequences returning <95% homology were manually checked to further eliminate corrupt sequences. The remaining sequences from all clone libraries were aligned using MUSCLE (Edgar, 2004) and a distance matrix was generated using the MEGA 4.0 software pack (Tamura *et al.*, 2007). Sequences were assigned to operational taxonomic units (OTU) using DOTUR (Schloss and Handelsman, 2005) and further analysis was carried out using SONS (Schloss and Handelsman, 2006).

The statistical significance of the observed changes was tested using a test developed for analysis of cDNA clone libraries (Audic and Claverie, 1997), similar to its use in the Library compare section of the Ribosomal Database Project-II website (Maidak *et al.*, 2001).

Phage-host systems

Isolation of bacterial strains—A fresh biomass sample from day 406 was diluted and spread over several Luria–Bertani (LB) agar plates. The plates were incubated at 37 °C for several days and single colonies were then picked manually. Streaking on LB agar was then used to isolate strains from these colonies. Streaking was repeated 2–3 times to ensure isolation. A total of 68 strains were isolated using this method. Isolated strains were kept under -80° C in 25% glycerol for further studies.

Selection of bacterial hosts

Bacterial hosts were selected using the soft agar method as follows: each isolate was suspended in 4 ml sterile, soft LB agar (0.5% agar in LB medium, 50 °C), together with 100 μ l of filter-sterilized (0.2 μ m) biomass sample from day 420. The soft agar was then poured over a pre-warmed (37 °C) LB agar plate and incubated for 1–5 days at 30 °C. Plates were checked daily for plaque formation.

Host strain DNA sequencing

The host DNA was extracted using a MO-Bio UltraCLean microbial DNA isolation kit (MO BIO laboratories, Carlsbad, CA, USA) according to manufacturer protocol. Total DNA was amplified with a Mastercycler gradient thermocycler (Eppendorf, Westbury, NY, USA) by PCR using specific 16S rRNA gene primers for bacteria (forward primer, 8F (GGATCCAGACTTTGAT(C/T)(A/C)TGGCTAG), and reverse primer, 1512R (GTGAAGCTTACGG(C/T) TAGCT-TGTTACGACTT) (Ben-Dov et al., 2008)). Primers used in the PCR amplifications were obtained from Sigma-Genosys. Reaction mixtures included a 25 µl ReddyMix PCR Master mix containing 1.5 mM MgCl₂ and 0.2 mM of each deoxynucleoside triphosphate (ABgene), 1 pmol each of the forward and reverse primers, $2-4 \mu l$ of the sample preparation and water to make up the total volume to 50 ml. An initial denaturation-hot start of 4 min at 95 °C was followed by 30 cycles of the following pattern: 94 °C for 30 s, 53–56 °C for 40 s and 72 °C for 105 s. A final soak at 72 $^{\circ}$ C for 20 min concluded the reaction. PCR products were purified by electrophoresis through a 1.0% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator. The approximately 1.5 kb heterologous 16S rRNA DNA products were excised from the gel, and the DNA was purified from gel slice by using the Wizard PCR Prep kit (Promega). The gel-purified PCR products were sequenced using an ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS and an ABI model 373A DNA sequencer (Perkin-Elmer, Waltham, MA, USA).

Changes in PFU concentrations

Thawed biomass samples from 24 time points were centrifuged (12 100 r.c.f., 1.5 ml per sample) for 1 min. Supernatant was filtered (0.2μ m) and 100 μ l aliquots were frozen ($-80 \,^{\circ}$ C) until further use so as to further minimize the freeze-thaw cycles. To maximize phage attachment, 200 μ l of overnight culture of each isolated host was mixed with 20 μ l biomass filtrate from each time point and incubated for 30 min at 37 °C in a rotary shaker. The mixtures were then used as inoculum for the top agar procedure and plaques were counted to estimate changes in PFU concentration over the study period for each of the host isolates.

Quantitative PCR

Total DNA samples prepared for clone library construction were diluted in double distilled water to 4–8 ngµl⁻¹ for use in quantitative PCR (QPCR). Forward primer PHS12F (5'-GAAAGCGGGGGACC TTTCG-3') was designed for this study for specific amplification of 16S rRNA gene sequence of host H12 and several sequences belonging to OTU 4. Reverse primer was universal 16S rRNA gene primer 341R (5'-CTGCTGCCTCCCGTAGG-3'). The reaction targeted a 165 bp region between nucleotides 176 and 341. The primer pair was tested by carrying out a PCR reaction on DNA from all 18 hosts and was found to be specific for H12 (results not shown).

Quantification of bacterial DNA was carried out in the ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using Absolute QPCR SYBR Green ROX Mix (ABgene) in a 96–well optical plate. The PCR reaction consisted of 10 ml Absolute QPCR SYBR Green ROX Mix, 150 nM each forward and reverse primers, and 5.0 ml of DNA template, in a total volume of 20 ml. Thermal cycling conditions were as follows: 2 min at 50 °C, 15 min at 95 °C, followed by 40 rounds of 15 s at 95 °C and 1 min at 60 °C. A dissociation protocol, testing dissociation of the PCR products from 60 to 95 °C was added after thermocycling to verify that the primer pair employed produced a single specific product. As standards for the calibration curves used for quantification, 16S rRNA gene fragments amplified from the total DNA of host H12 were used in seven serial dilution points (in steps of tenfold). A no template control was also included. Detection was based on fluorescence resonance energy transfer with a SYBR green fluorophore. The cycle number at which the signal was first detected correlated with the original concentration of the DNA template, whereas the starting copy number of amplicons was inversely proportional to the real-time threshold cycle. Standard curves were obtained by plotting the threshold cycle value of each tenfold dilution series of H12 16S rRNA. Standards and environmental samples were simultaneously assayed in triplicates or quadruplicates. The ABI prism 7000 Sequence Detection System and SDS Software were used for data analysis.

Database submission of host sequences

Host 16S rRNA gene sequences were submitted to the GenBank database, accession numbers GQ254271–GQ254288.

Results

In order to better understand the following results, it is necessary to understand the sampling regime and work flow. A more detailed description is given in the Materials and methods section.

Biomass samples were drawn over a period of 462 days, from the aeration basin of a full-scale membrane bioreactor for the treatment of industrial wastewater. Aliquots from each sample were stored at -80 °C to be used later for DNA extraction and clone library construction. The same samples were subsequently used for PFU counts on bacterial strains isolated towards the end of the study period. This enabled a retrospective study of changes in the abundance of bacteriophage-infecting specific hosts.

Changes in the relative abundance of bacterial groups as a function of time

Clone libraries were constructed for 19 of the 25 time points sampled over the course of the study. After elimination of chimeras and unusable sequences, the 19 clone libraries contained a total of 1588 sequences with over 700 bp alignment. The number of sequences per library was between 75 and 91 (average 84). The 1588 sequences were grouped into 255 OUTs based on 99% sequence homology, or 159 OTUs based on 97% sequence homology. Numbering of the OTUs was assigned based on the order of appearance of sequences in the clone libraries. Analysis of the sequences obtained showed frequent changes in the bioreactor's microbial community composition. The relative abundance of an OTU at different time points was calculated as the ratio between the number of



Figure 1 Changes in relative operational taxonomic unit (OTU) abundance as a function of time. Changes in relative OTU abundance (99% similarity) in the clone libraries are shown for four bacterial groups. (a) OTU 4, closely related by 16S rRNA gene sequence to the genus *Alcaligenes* in the class *Betaproteobacteria*. (b) OTU 17, approximately 90% 16S similarity to the genus *Trupera* in the phylum *Deinococcus-Thermus*. (c) OTU 19, closely related to the genera *Aquamicrobium* in the class *Alphaproteobacteria*. (d) OTU 45, closely related to the genus *Salinibacter* in the phylum *Bacteriodetes*. (*) Significant difference in relative OTU abundance between two adjacent time points (P < 0.05).

sequences in that OTU in the clone library representing that time point and the total number of sequences in the library. Changes in the relative abundance of four OTUs are shown in Figure 1. Table 1 gives the number of sequences in each clone library for two of these OTUs.

Many of the observed changes in OTU relative abundance could not be correlated to the changes in measured parameters from either the bioreactor or the incoming wastewater. Phage activity was proposed as an alternative hypothesis explaining some of these changes. To test this hypothesis, bacterial strains were isolated from the reactor's biomass and tested as potential hosts for bacteriophage found in the reactor.

Bacterial hosts isolation and identification

A total of 68 bacterial strains were isolated from a biomass sample obtained on day 406 of the

Table 1 Relative OTU abundance and PFU counts over time

Day	No# Seq OTU 4	H12 [PFU (±StDev)]	H17 [PFU]	No# Seq OTU 19	H3 [PFU]	H8 [PFU]	H14 [PFU]	H15 [PFU]	H16 [PFU]	H18 [PFU]
0	4	77400 (±14,990)	0	1	0	0	0	0	0	50
14	ND	56900 (±2120)	0	ND	0	0	0	0	0	0
35	1	$11800 (\pm 1400)$	0	0	0	0	0	0	0	0
49	ND	$50(\pm 70)$	0	ND	0	0	0	0	0	0
63	9	2450 (±350)	0	0	0	0	0	0	0	0
77	ND	$6650(\pm 640)$	0	ND	0	0	0	0	0	0
92	7	ND	ND	2	ND	ND	ND	ND	ND	ND
105	ND	17500 (±2120)	300	ND	30000	10000	0	800	400000	0
119	4	9100 (±1560)	0	0	10000	0	0	0	23000	0
134	ND	750 (±70)	0	ND	950	0	0	0	6650	0
147	5	$100(\pm 0)$	0	1	100	0	0	0	1750	100
161	ND	0	1250	ND	950	100	0	0	7000	0
175	0	1400 (±140)	0	1	100	0	0	0	1200	100
217	1	0	0	2	0	400	0	0	2250	0
245	1	0	0	0	0	0	0	100	0	0
273	6	0	0	2	0	0	0	0	0	0
295	3	0	14100	6	0	250	2450	19233	0	0
341	1	5250 (±1630)	0	0	0	250	0	0	0	0
350	0	$1100(\pm 140)$	0	0	0	350	0	0	0	0
378	1	500 (±420)	0	0	1350	500	800	0	3400	200
393	0	$150(\pm 70)$	23000	1	19800	4150	7000	1750	21000	9900
406	0	850 (±70)	25000	0	13850	15500	10100	850	68000	7800
420	0	700	3000	0	11350	11500	2600	1500	37000	4150
448	1	$50(\pm 70)$	133000	3	4450	66000	52000	50	11250	12150
462	1	600 (±140)	14000	2	18800	4200	4000	35000	8000	2100

ND = not determined.

sampling period. Eighteen of the isolates gave rise to clear plaques using the soft agar method when tested with a filtered biomass sample obtained on day 420, indicating the presence of lytic phages targeting these isolates in concentrations exceeding 10 PFU ml⁻¹. These isolates are referred to as strains H1–H18. Comparison of 16S rRNA gene sequences from strains H1-H18 to sequences from the clone libraries returned over 98% homology with at least one cloned sequence for 12 of the 18 strains. The 16S rRNA sequences of strains H5, H13 and H17 shared over 99% homology with a single OTU (designated OTU 4), whereas strain H12 shared over 98% homology with the same OTU. Sequences contained in OTU 4 were found in 14 of the 19 clone libraries, thus making it the third most abundant in the sequence collection. The 16S rRNA gene sequences of strains H3, H8, H14, H15 and H16 shared over 99% homology with OTU 19, whereas strain H18 shared 98% sequence homology with the same OTU. OTU 19 contained sequences from 10 different time points, making it the eighth most abundant OTU. Partial 16S rRNA gene sequences, obtained for strains H1-H18, were grouped into three major clades, which corresponded to the bacterial families Pseudomonadaceae. Alcaligenaceae and *Phyllobacteriaceae*. Figure 2 shows the phylogenetic relationships between isolates H1–H18, representative sequences from the clone libraries and the nearest matching sequence from isolated strains deposited in Gene-Bank (NCBI).



Figure 2 Neighbour-joining tree of host sequences. Branch length represents phylogenetic distance between 16S rRNA gene sequences of the 18 isolated hosts (H1–H18), representative sequences from the clone libraries (M1–M19) and closest matched sequences of isolated strains from the NCBI BLAST search.



Figure 3 Changes in bacterial and viral abundance in the bioreactor as a function of time: operational taxonomic unit (OTU) 4. (a) Changes in plaque-forming unit (PFU) counts of hosts H12 (\bullet) and H17 (\bigcirc) as compared with the changes in relative abundance (% of clone library) of sequences in OTU 4 (97% similarity) (\diamond). (b) Changes in copy number of 16S rRNA gene sequences related to host H12 and some sequences from OTU 4 (\diamond). Copy number determined by quantitative PCR (QPCR). Error bars represent s.d. for three or four reactions.

Changes in PFU concentrations as a function of time Of the 18 hosts, H1, H7, H11 and H13 did not yield plaques when used as the lawn for phage tests with any of the frozen biomass samples. Host 5 gave rise to plaques only when tested with a sample obtained on day 341 of the study. Lytic phage infecting one or more of the remaining 13 hosts were found at all examined time points. At least three susceptible hosts were found for each time point. PFU counts for eight of the hosts are presented in Table 1. The highest PFU concentration was found for host H16 on day 105, with over $400\,000\,\text{PFU}\,\text{ml}^{-1}$. The results for isolates related to family *Pseudomondaceae* were poorly reproducible, with the plaques forming on these isolates being unclear and difficult to enumerate. These isolates will not be further discussed.

Changes in PFU concentration as a function of time for two of the hosts are presented in Figure 3. Panel A in Figure 3 shows PFU counts for hosts H12 and H17, both related to the family Alcaligineaceaea. Active phage for host H12 were found in 19 of the 24 samples. Six separate infection cycles were observed for H12 during the study period. The highest measured concentration, $77\,000$ (±15000) $PFU ml^{-1}$, was found on day '0', obtained less then a month following initial reactor operation. This appears to be the peak of an infection cycle, with PFU concentrations gradually decreasing to $<100\,PFU\,ml^{-1}$ over the next 49 days. A second cycle was observed on day 63, with a peak concentration of 17 500 (\pm 2100) PFU ml⁻¹ detected on day 105. The cycle ends by day 161, when PFU counts return to below 100 PFU ml⁻¹. A single burst of 1400 (\pm 150) PFU ml⁻¹ was detected on day 175.



Figure 4 Changes in bacterial and viral abundance in the bioreactor as a function of time: operational taxonomic unit (OTU) 19. (a) Changes in plaque-forming unit (PFU) counts for hosts H3 (\odot) and H15 (\bigcirc) compared with the changes in the relative abundance (% of clone library) of sequences in OTU 19 (99% similarity) (\diamond). (b) Changes in PFU counts for hosts H8 (\odot) and H16 (\bigcirc) compared with the changes in the relative abundance (% of clone library) of sequences in OTU 19 (99% similarity) (\diamond).

A fourth, smaller cycle with a peak of 5200 (± 1600) PFU ml⁻¹ begins on day 341, followed by two smaller bursts at approximately 60 days intervals. The five infection cycles found for strain H17 demonstrate an opposite trend, with peak PFU concentration rising with each subsequent cycle. However, the first three peaks are only represented by a single time point, therefore indicating that the length of the infection cycle was shorter than the sampling interval.

Figure 4 shows changes in PFU concentrations for host pairs (H3, H15) and (H8, H16) in panels A and B, respectively. The 16S rRNA gene sequences for these hosts differed by < 1% (Figure 2). For the four hosts, different plaque sizes and morphologies were noted between different infection cycles, and sometimes within the same sample, possibly indicating the presence of several phage types infecting a single host. The counts presented are total PFU, with no regard to plaque morphology. Again, a pattern of infection cycles may be observed, with different strains showing peak values at different time points. Here, different peak values in OTU 19 abundance correspond to peak PFU counts from different strains.

QPCR

To provide a quantitative estimate for the observed changes in OTU relative abundance, QPCR analysis was carried out by targeting 16S rRNA gene sequences, which are related to strain H12 and some of the sequences included in OTU 4. Results of the QPCR analysis are presented in Figure 3b. The results show peaks in H12 16S rRNA gene copy numbers on days 63, 147 and 273, similar to the timing of major peaks observed for OTU 4. Towards the end of the study period there was a constant rise in gene copy numbers that was poorly reflected in the clone libraries (one homolog sequence in each of the clone libraries from days 448 and 462). H12-like 16S rRNA gene sequences were found in the bioreactor at all time points, with copy numbers spanning a range of 2 orders of magnitude, from 1.58×10^{6} on day 119 to 2.2×10^{8} on day 462. A large change in copy numbers is observed between days 245 and 273. This change is reflected by a peak in OTU 4 relative abundance on day 273. H17 PFU counts rise in the following period from under 100 PFU ml^{-1} on day 273 to over 14 000 PFU ml $^{-1}$ on day 295. H12 PFU counts on day 295 are below 100 PFU ml^{-1} , rising to over 5000 PFU ml^{-1} on day 341. A shortage in DNA template prevented the application of QPCR to hosts related to OTU 19.

Discussion

Bacteriophages were previously shown to be present in high numbers in wastewater treatment systems (Ewert and Paynter, 1980; Hantula et al., 1991; Wu and Liu, 2009). However, little information has been published regarding their population dynamics and their interaction with the microbial community. Moreover, phage impact on specific hosts in this environment, or indeed in most other environments, has not experimentally been demonstrated. In this study, bacteriophages were shown to be present and active in a bioreactor treating high-strength chemical industrial wastewater. This study also demonstrates the temporal dynamics of phage infecting specific bacterial strains, with the abundance of specific phage generally low, interposed by periods of high PFU counts. Changes in the abundance of bacterial groups phylogenetically related to the hosting bacterial strains were correlated to the observed changes in phage abundance. Most long-term studies enumerate total phage or viral-like particles using transmission electron microscopy (Jiang and Paul, 1994; Mathias et al., 1995) epi-fluorescence microscopy (Otawa et al., 2007) and more recently FACS analysis (Lymer et al., 2008; Boras et al., 2009). Although these methods work well for comparing total bacteria and total phage numbers in a given environment, they provide no data regarding specific phage-host interactions. For that purpose, one must isolate and study specific phagehost systems, as was done in this study and in a small number of other studies covering shorter time periods (Hantula et al., 1991; Comeau et al., 2005; Clokie *et al.*, 2006). As phage abundance depends on the concentration of its specific host, it is preferable to isolate strains that are sufficiently dominant in the studied environment. This is often not an easy task, as the majority of microbial species are not readily cultivable under routine laboratory procedures. This phenomenon, referred to as 'the great plate count anomaly', is most prominent in sea water, where only 0.001-0.01% of bacteria are said to be readily cultivable (Amann et al., 1995). A less often cited number from the same source is 5–15% of bacterial cells found in activated sludge being readily cultivable. Such relative ease of cultivation makes the activated sludge and other intensive wastewater treatment processes attractive model environments for phage ecology studies. Indeed, in this study, the two host groups related by 16S rRNA gene sequence to Alcaligenaceae and Phyllobacter*iaceae* (Figure 2) were closely related to the third and eighth most abundant OTUs in the clone libraries, out of 255 observed OTUs at 99% homology. This result enables a rare glimpse into the population dynamics of abundant bacterial species or groups and their specific predators in a complex chaotic microbial community.

Two main patterns can be drawn from the data presented here. One is the apparent correlation between peaks in phage concentrations in the reactor and peaks in OTU relative abundance in the clone libraries. A Pearson's correlation test for OTU 4 and PFU counts for host H12 returned the highest ρ -value when PFU counts were correlated with OTU abundance of 14 days earlier ($\rho = 0.77$). A similar correlation for OTU 19 and the sum of PFU counts for all related hosts returned a less conclusive result ($\rho = 0.33$), possibly due to the low representation of sequences related to OTU 19.

The OTU relative abundance is not a quantitative measure. However, the apparent synchronization between changes in OTU abundance and changes in gene copy number measured by QPCR shows the ability of clone libraries to reflect changes in bacterial community composition, at least for the more abundant OTUs.

The correlation between host abundance and PFU counts is predicted by the 'killing the winner' hypothesis (Thingstad and Lignell, 1997; Thingstad, 2000), an idealized Lotka–Volterra-type model, stating that a rise in abundance of a bacterial species is followed by a rise in abundance of its specific phage. This is similar to other predator-prey models in ecological theory (Jansen, 1970; Chase et al., 2002). However, the highest PFU count in this study is 4×10^5 , an order of magnitude lower than the lowest gene copy numbers detected by QPCR. Thus, the number of viruses detected does not appear to be high enough to elicit drastic reduction in bacterial numbers. It is therefore not clear from the data presented here, whether the fall in bacterial numbers is caused by phage activity or by external, unknown factors.

It may be argued that all PFU counts recorded in this study are likely gross underestimations, as only detached phage particles from the biomass supernatant were counted. Active phage within bacterial aggregates, or attached to particles larger than $0.2\,\mu$ m, were thus not considered. As it was impossible to estimate the true size of the viral population in this study, an equal proportion was assumed to be lost for all samples, meaning that changes in PFU counts reflect changes in total viral population.

Aggregates account for the majority of bacteria in the bioreactor, and are likely the site of much viral activity. It is virtually impossible to release all the phage particles from the dense biomass typical of wastewater bioreactors. However, pre-treatment of the biomass to mechanically disrupt the bacterial aggregates was shown to increase bacterial counts in activated sludge by an order of magnitude (Ziglio et al., 2002). Wu and Liu (2009) show a rise in total viral counts for dense biomass from activated sludge by combining several elution steps. A similar approach could be employed in future studies to maximize phage counts. Freeze-thaw cycles and thermosensitvity are also expected to negatively affect some phage species, as was reported in an earlier study (Hantula et al., 1991). Possible effects of freezing were also observed in this study, as a decrease in phage counts occurred following the freezing of the samples. The combined effect of these mechanisms may explain some of the discrepancy between low phage counts and high bacterial concentrations.

A second pattern that emerges upon comparing the data is a succession of PFU peak values for phylogenetically related strains. Examples of this can be seen in Table 1 and Figure 3, wherein peaks in PFU counts for strain H17 on days 161 and 295 are followed by peak PFU counts for strain H12 on days 175 and 341.

A similar pattern can be seen for PFU counts in Figure 4, towards the end of the study period. As a change in phage counts necessarily reflects a change in the abundance of its specific host, this pattern indicates changes in the micro-diversity within the microbial population. The most stable conditions in the reactor were recorded during the last 70 days of the study period, with no known external influences accounting for the observed alterations in phage or bacterial species abundance. The pattern observed may therefore be interpreted as reflecting competition for resources between closely related subspecies, mediated by specific phage predation. With several closely related species, sharing metabolic traits and favorable conditions, competing for an available niche in the environment, a 'winner' will ultimately become the most abundant species. This 'winner', having reached a certain threshold concentration, becomes susceptible to specific phage predation, thereby losing its competitive advantage. This opens the niche to a new 'winner', and the process repeats itself. The ousted competitor remains in the environment in low numbers, avoiding predation, until concentrations of its specific phage decline, thus allowing its return into the competition. This is the mechanism suggested by the 'killing the winner' paradigm (Thingstad and Lignell, 1997; Thingstad, 2000), but with selection between closely related strains of the same species, separated by their susceptibility to different bacteriophage in the environment. These results suggest that, by reducing the abundance of dominant strains, phage selective predation promotes not only the total microbial diversity (Weinbauer and Rassoulzadegan, 2004) but also the micro-diversity (Schloter *et al.*, 2000), meaning that several closely related sub-species can co-exist in the same environment without one outcompeting the other.

Antagonistic host-parasite co-evolution between a phage and its hosting bacterium has been demonstrated in laboratory experiments and suggested to have a major role in ecological environments (Buckling and Rainey, 2002). The underlying principal is that a host and parasite co-existing in the same environment need to undergo an evolutionary arms race to maintain their ability to survive in the environment, a process that has also been termed 'Red queen hypothesis' (Lythgoe and Read, 1998). Based on the results presented, it is not possible to determine whether or not such a process takes place in the studied bioreactor. As all hosts were isolated towards the end of the study period, an arms race should have resulted in reduced susceptibility to phage from the beginning of the study, similar to the results obtained by Buckling and Rainey (2002). This is clearly not the case for host H12, for which the highest PFU counts were found in the first and second time points. The same may be said for hosts H3 and H16, with the highest PFU counts on day 105. On the other hand, there is a general increase in phage activity for most hosts from day 393 onwards, that is, following the isolation of the hosts. This is consistent with co-evolution, but may also be the result of novel strains recruited from the surrounding environment and the propagation of bacteriophage specific to these strains.

Phage resistance is often associated with reduced fitness (Buckling and Rainey, 2002), so that a resistant mutant may not survive the intense competition for resources in the bioreactor. As free phage particles are constantly washed out of the bioreactor, whereas bacteria are retained by the membrane, low numbers of susceptible hosts are likely to persist in the system after their specific phage were mostly removed. These hosts, not having paid the price associated with resistance, are likely to outgrow any resistant strains that evolved. The importance of co-evolution in bioreactors and similar ecological systems is therefore unclear and needs to be studied further.

Microbial diversity was previously shown to be a key factor in the functional stability and resilience to perturbation of microbial systems (Briones and Raskin, 2003; Girvan *et al.*, 2005). It is therefore suggested that phage specific predation has a key role in maintaining the stable activity and performance of wastewater treatment bioreactors.

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In this study, phage predation is shown to affect microbial community composition in a wastewater treatment bioreactor, with possible implications for other environments and environmental processes.

These findings represent a first attempt to track the stochastic behavior of specific phage-host systems in a complex environment over a prolonged time period. Frequent changes in both OTU abundance and PFU counts are observed throughout the study period. The patterns emerging from the data are consistent with the 'killing the winner' hypothesis. However, because of low phage counts, the possibility that observed changes in OTU abundance are caused by factors other than phage predation cannot be ruled out.

One interesting conclusion from these results is that, while a system may appear to be in steady-state (judged commonly in wastewater treatment processes by a relatively stable performance in regard to residual organic content and total biomass concentration), a stable microbial community composition is not likely to evolve, as no single bacterial strain can dominate a given niche indefinitely. Based on that conclusion, phage activity may have a more central role in wastewater treatment systems than previously suggested, maintaining the microbial diversity necessary for functional redundancy in these important systems.

Conflict of interest

The authors declare no conflict of interest.

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