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Analysis of environmental variables on population dynamic change of *Haliscomenobacter hydrossis*, the bulking causative filament in Macau wastewater treatment plant

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ABSTRACT

Macau Peninsula wastewater treatment plant (WWTP) is experiencing filamentous bulking in recent years, which is caused by overgrowth of filamentous bacteria of *Haliscomenobacter hydrossis*, thus interfering the settling of activated sludge. Previous studies using molecular techniques have been widely used for identifying and quantifying the dominant filaments. However, the mechanisms caused by various environmental variables have not yet been completely understood to form the deterministic cause–effect relationship. In this study, principal component analysis (PCA) and generalized linear model (GLM) were used to analyze various environmental variables on the population dynamic changes of *H. hydrossis* identified and quantified as the dominant bulking causative filament using quantitative real-time PCR (qPCR). Our results showed that the qPCR was successful for identifying and quantifying the *H. hydrossis*. Correlation analysis indicated that ammonia nitrogen sources and sludge volume index (SVI) have strongly positive correlations with *H. hydrossis*. PCA can explain 75.21% of the variance from the total data in PC1-PC4 and GLM revealed a unimodal relationship between the overall *H. hydrossis* gene copies number and three environmental variables. Besides, the quadratic increases in the number of *H. hydrossis* copies were observed with the increasing gradient of SVI, TN, and NH_3-N, suggesting that *H. hydrossis* played an important role in filamentous bulking of the WWTP.

Keywords: *Haliscomenobacter hydrossis*; Filamentous bulking; qPCR; Correlation analysis; PCA; GLM

1. Introduction

Filamentous bulking is the most common solids settling problem in wastewater treatment plants (WWTP), which is caused by the excessive growth of filamentous bacteria outside the flocs, thus interfering the settling of activated sludge [1–3]. Bulking results in high concentration of suspended solids in the effluent and subsequently loses activated sludge in the aeration basin, leading to the deterioration of water quality and wastewater treatment process. It has been reported that over 50% of the wastewater treatments in US experience bulking [3]. Proliferation of filamentous bacteria depends on the seasonal changes and

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operational conditions [4], from which the preventive and control measures were developed. Low dissolved oxygen and temperature, high sludge retention time, and continuous mixed operations were cited as the parameters associated with filaments' growth [5]. However, these measures are not based on the knowledge of the physiology or kinetics of a specific type of filaments, thus bulking controls remain on trial and error basis.

In recent years, Macau Peninsula WWTP, the main wastewater treatment system in Macau, is experiencing filamentous bulking, with a high level of filamentous bacteria, dominated by *Haliscomenobacter hydrossis* that was identified and quantified by Ion Torrent deep sequencing, with the highest concentration of around 5.5×10^9 copies g sludge^{-1} on 3 December 2012. Deep sequencing, such as ion torrent, showed superior on profiling microorganism community and structure [6–9] and could be helpful in designing and evaluating oligonucleotide probes and primers [10]. However, compared to qPCR, ion torrent costs much more, and the database analysis which is the after-process needs a lot of compute devices, based on which, real-time methods could be developed for monitoring various types of species. Recent studies using real-time PCR has been successfully applied to quantify various filamentous species including Type 021N [11], *Microthrix parvicella* [12,13], *Thiothrix* spp. [14,15], suggesting that real-time PCR could be accurate enough to quantify population abundance in bulking activated sludge.

H. hydrossis is a filamentous microorganism commonly present in WWTPs and was reported as a major bulking causing filament in the world [16,17]. *H. hydrossis* ranks ninth in the number of predominance [3]. It belongs to the *Cytophaga/Flexibacter*-group of the *Bacteroidetes*, and stains Gram negative and Neisser negative. FISH probe based on the nucleotide sequence of a *H. hydrossis* strain in the DSMZ collection has been developed and applied to activated sludge samples.

In Macau Peninsula WWTP, the conditions for an abundant development of *H. hydrossis* and the main water parameters that give rise to bulking sludge were not completely understood. Environmental factors have significant effects on microorganism abundance in WWTP, and the sensitivity of the microorganism also has specific preferences. At present, it has developed a variety of statistical model used to describe their interest in the relationship between the quality and biomass.

Principal component analysis (PCA) can be used for multicollinearity data on multivariate statistical methods. The PCA is to use an orthogonal transformation, to convert observations of possibly correlated variables into values of uncorrelated variables called PCs, thus reducing the complexity of multidimensional system by maximization of component loadings variance and elimination of invalid components. PCA has been used alone or in combination with other methods, such as generalized linear model (GLM), to model species abundance and environmental factors. PCA was used to find out the principal component from the variables. Then, regression analysis was performed between response variable and selected principal components. It is followed GLM to check if the microorganism abundance could be explained by environmental variables, and to use for further prediction.

GLM was a flexible generalization of ordinary linear regression that allows for response variables that have error distribution models other than a normal distribution. The GLM generalized linear regression by allowing the linear model to be related to the response variable via a link function and by allowing the magnitude of the variance of each measurement to be a function of its predicted value. Compared to the general linear regression based on normal distribution assumption, and directly to simulate the average, GLM promotes to general linear regression model in two ways: First, it allows the response variable for abnormal distribution; second, it can simulate the function of the average. It could help for understanding of the relationships between species and environmental variables in depth [18].

To better understand the relationship between the bulking levels expressed as sludge volume index (SVIs) and the dynamic change of *H. hydrossis*, and their correlation among various water parameters that cause bulking, real-time PCR was applied in this study for identifying and quantifying the population of *H. hydrossis* in the samples collected weekly from July 2012 to May 2013 in Macau WWTP. Correlation analysis was performed to detect the simple relationships between every two parameters. For the possible multicollinearity, PCA was performed to determine the main explaining variables, followed by GLM that was applied to predict the *H. hydrossis* from the significant environmental variables.

2. Material and methods

2.1. Macau Peninsula WWTP, sampling and parameter measurement

Macau Peninsula WWTP (113°33′12″ E in longitude and 22°12′12″ N in latitude), located in the east part of Macau peninsula, is the largest WWTP in Macau. The plant is a typical municipal WWTP, which has the total designed capacity of 356,000 cubic meters

per day, and currently treats around 80% of the total volume of wastewater in Macau. It is operated as conventional activated sludge treatment process, i.e. continuously completely mixed reactor, which is susceptible to filamentous bulking.

During the study period, activated sludge samples were collected weekly from the aeration basin of Macau Peninsula WWTP from July 2012 to May 2013. They were kept at 4℃ and immediately transferred to the laboratory. Gram staining and Neisser staining were performed to study the morphology of *H. hydrossis* [3]. The samples were centrifuged at 3,000 rpm for 5 min to remove the supernatant and kept in −80℃ freezer until DNA extraction.

A total of nine water quality parameters, including 5-d biological oxygen demand (BOD_5), chemical oxygen demand (COD), mixed liquid suspended solids (MLSS), pH, ammonia nitrogen (NH_3-N), total nitrogen (TN), total phosphorus (TP), food-to-mass (F/M) ratio, and SVI were measured, according to the standard methods [19]. The F/M ratio was determined by the multiplication of influent flow and the BOD. The analysis was performed within 24 h after sampling.

2.2. DNA extraction

The sludge sample was re-suspended at the initial concentration, 200 ml of which was centrifuged at 3,000 rpm for 10 min to obtain the pellet of about 1,000 mg. The DNA from the pellet was extracted using UltraClean® Soil DNA Isolation Kit (Q-Biogene, CA) and then quantified by Qubit 2.0 instrument using the Quant-iT™ dsDNA BR kits (Invitrogen, Carlsbad, CA) prior to storage at −20℃.

2.3. Primer development

There was no specific primer in the literature for the 16S rDNA gene of *H. hydrossis*. Therefore, the new primers (Table 1) targeting *t*he 16S rRNA genes of some of the *H. hydrossis* DSM 1100, known as *H. hydrossis*, were designed by the Invitrogen Trading (Shanghai) Co., Ltd., a global company targeting gene products and services. The new designed primers were checked in BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The BLAST programs compared a query sequence to all sequences in a specified database.

2.4. PCR

Genomic DNA templates of sludge sample were amplified by GeneAmp® PCR system 9700 (Applied Biosystems, CA) to demonstrate the presence of *H. hydrossis* using specific primer sets (Table 1). Primers specificities to the selected sequences in this study were checked in BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Each PCR mixture contained 1 μL of DNA template solution (totally 50 μL PCR), 5 μL of 10 × PCR Buffer ($MgCl_2$ plus), 8 μL of the dNTP mixture, 1 μL of each primer, and 0.5 μL of *TaKaRa Taq*™, 20 mM Tris–HCl (pH 8.0) and was adjusted to a final volume of 50 μL with sterile water (Sigma, USA). The PCR was performed as follows: 94℃ for 3 min, 94℃ for 30 s, 35 cycles at 50℃ for 30 s, 72℃ for 8 min, and a final extension step at 4℃ for holding. Gel electrophoresis was run to confirm the presence of *H. hydrossis* in the samples. All PCR products were separated on 2% agarose gels and observed on Molecular Imager ChemiDoc XRS System (Bio-Rad, USA) after staining with 6× loading dye for 20 min.

2.5. Real-time qPCR

Real-time qPCR were performed in ABI 7500 Real-time PCR system (Applied Biosystems, CA). All reactions were carried out in a total volume of 20 μL, containing 10.4 μL SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus), including *TaKaRa Ex Taq* HS, dNTP Mixture, Mg^{2+}, Tli RNaseH, SYBR® Green I, plus ROX Reference Dye Ⅱ (DRR420, TaKaRa Biotechnology, China), 0.8 μL forwards primers and 0.8 μL reverse primers (Table 1), 6 μL deionized water and 2 μL DNA templates. The thermal protocol for *H. hydrossis* was a 10-min step at 95℃, followed by 40 cycles of 15 s at 95℃, 2-min at 55℃, and 1-min at 60℃ in second step, and finally dissociated a 15-s at 95℃, 1-min at 60℃, and a 15-s at 95℃ in the third step. Fluorescence was measured at the end of each cycle at 72℃ through channel F1 (530 nm) and a heating rate of 20℃ s^{-1}. All samples were amplified in triplicate. After qPCR amplification, fluorescent melting curve analysis was

Table 1
Primers used for qPCR and PCR assays

Target	Primer	Sequence (5′-3′)	DNA length	Reference
Haliscomenobacter hydrossis	HHY F HHY R	TTCTGGCGCTGAAGGATGAG GTGTCTCAGTACCCGTGTGG	118 bps	This study

performed by gradually increasing the temperature from 72 to 95°C at a rate of $0.1^{\circ}s^{-1}$. A correlation between the gene copy numbers and the threshold cycle number (Ct) (the cycle number at which the fluorescence exceeds the threshold) can be obtained.

2.6. Construction of standard curves for H. hydrossis copy number determination

A method was performed using absolute quantification in the form of a plasmid belong to the target gene of *H. hydrossis* in the wastewater sludge. For quantification of gene-specific 16S rRNA using quantitative real-time PCR, the quantification method was achieved using a standard curve.

With the molecular weight of the plasmid and insert known, the mass concentration of the plasmid can be measured and then converted to the copy concentration as Eq. (1) [20]:

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}\ (\text{copies mol}^{-1}) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660\ (\text{g mol}^{-1}\text{bp}^{-1})} \quad (1)$$

After the qPCR analysis, the Ct value of gene specific was evaluated by the standard curve. Then, the $\log_{10}$ value can be achieved. The copies of per wastewater sludge (copies g^{-1}) can be calculated by the following Eq. (2) [21]:

$$\text{Copy numbers (copies g}^{-1}) = (\text{MQ} \times \text{C} \times \text{VD})/(\text{S} \times \text{V}) \quad (2)$$

where MQ (copies) is the quantitative mean of the copy number according to the standard curve, C (ng μL^{-1}) is the DNA concentration of each sample, VD (μL) is the dilution volume of extracted DNA, S (ng) is the DNA amount for analysis, and V (g) is the wastewater sludge mass for DNA extraction.

As there was no plasmid in the literature, the plasmid containing *H. hydrossis* 16S rDNA gene was synthesized by Invitrogen Trading (Shanghai) Co., Ltd. and used as the standard. Standard curves and the detection limits for qPCR were established using 10-fold dilutions of the extracted pure culture DNA, with the concentrations ranging from 3.0×10^{-2} to 3.0×10^{-6} ng μL^{-1}, and corresponding primers (Table 1) were applied to the plasmid.

2.7. Correlation analysis

Correlation coefficients were calculated using Pearson's statistic (R_p) for standardized strain mean data (i.e. *z*-scores) and Spearman's statistic (R_s) for strain mean rank data. The latter method was employed because some data were abnormal distribution, the degree of relationship may not be indexed appropriately by the simple Pearson correlation [22].

2.8. Principal component analysis

PCA is a multivariate statistical method which to use an orthogonal transformation to convert observations of possibly correlated variables into values of uncorrelated variables called PCs, thus reducing the complexity of multidimensional system by maximization of component loadings variance and elimination of invalid components. PCs are expressed by the following Eq. (3):

$$PC_i = a_{1i}V_1 + a_{2i}V_2 + \cdots + a_{ni}V_n \quad (3)$$

where PC_i is principal component *i* and a_{ji} is the loading (correlation coefficient) of the original variable V_i.

PCA among the water quality parameters, operational parameters, and *H. hydrossis* gene copies was carried out using PASW 19 software package (SPSS Inc.), to determine the factors influencing the filamentous bulking in Macau WWTP. Due to the wide ranges of data dimensionality and different units of measurements, standardized data through normalized transformation were required [23,24].

2.9. Regression analysis

Based on the results of PCA, GLM [25] was used to test for the significance of environmental preferences, but also for the effect and interaction of other external factors. *H. hydrossis* copies number was treated as the response variable and regressed against the other environmental variables. A generalized linear model with a Poisson error distribution was used to elucidate the pattern of specie abundance along the different environmental factors. Tests were performed using the *F*-statistic. CANOCO 4.5 for windows was used.

3. Results

3.1. Macau Peninsula WWTP

In recent years, the Macau Peninsula WWTP was reported to experience filamentous bulking with high SVI (>150 mL/g TSS) in the summers. The influent concentrations of COD, NH_3-N, TN and TP, and SVIs in 2011–2013 were showed in Figs. 1 and 2,

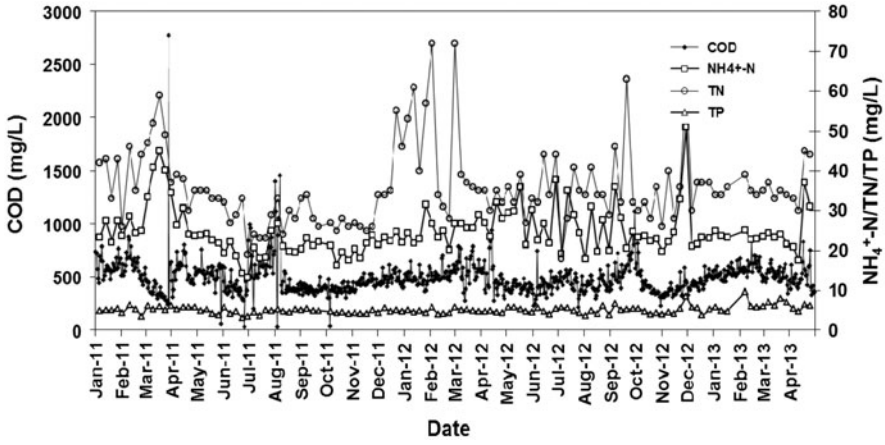


Fig. 1. Change of influent COD, NH_4^+-N, TN, and TP over 2011–2013.

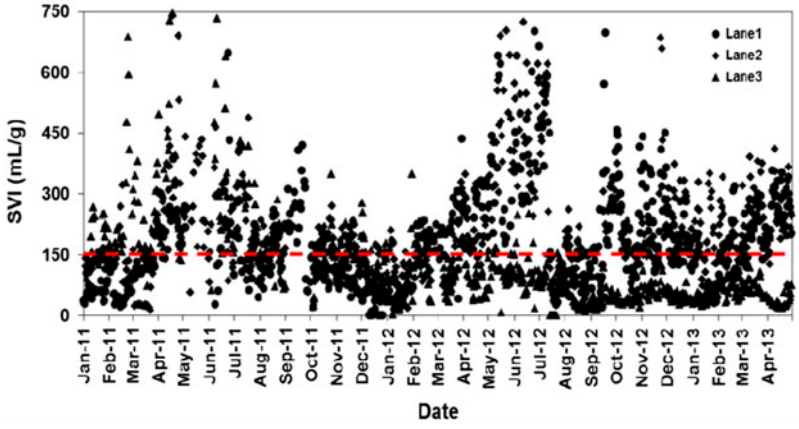


Fig. 2. Change of the SVIs over 2011–2013, lane 1, 2, 3 are three different lanes in WWTP, sampled in their aeration tank.

respectively. The SVIs exhibited a periodic pattern each year, with bulking occurred from March of 2011 to September of 2011 and October of 2013 to April of 2013, while non-bulking happened from October of 2011 to February of 2012 and July of 2012 to September of 2013. The highest SVIs were mostly in May–June in 2011 with the SVIs up to 3,000 mL/g, implying that high temperature favors the filamentous bulking.

3.2. Morphological analysis of the samples

Based on morphological analysis and staining, the abundance of *H. hydrossis* was observed in the samples. The *H. hydrossis* morphotype showed needle-like appearance or longer slightly bent filaments protruding from the flocs. Cells were surrounded by a sheath, and trichome was approximately 0 3–0.5 μm in diameter and 10–100 μm in diameter in length. They were stained Gram negative and Neisser negative. These morphology and characteristics were similar to those described by Jenkins et al. [3]. Survey using FISH probes [26] (data not shown here) confirmed the dominance of *H. hydrossis* in the samples. Other filamentous bacteria including *Thiothrix* spp., *Sphaerotilus natans*, "*Nostocoida limicola*" III, Type 021N, and Type 1851 were also observed using morphological analysis. These results were consistent with our previous Ion Torrent deep sequencing, showing that the *H. hydrossis* was the dominant filamentous species that may cause bulking.

3.3. PCR and qPCR

Though FISH has been widely used for monitoring *H. hydrossis* in the activated sludge samples, not much work has been done to apply PCR and qPCR for identifying and quantifying *H. hydrossis*. The PCR amplification results (Fig. 3) indicated the feasibility of using

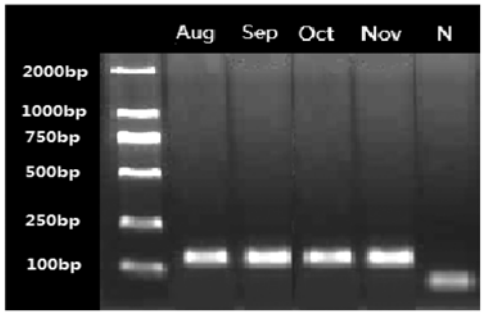


Fig. 3. PCR amplification results of *H. hydrossis* 16S rRNA on 8th Aug. 2013 (Aug), 28th Sep. 2013 (Sep), 12th Oct. 2013 (Oct), 23th Nov. 2013 (Nov), and the negative control (N).

primer HHY F and HHY R for detection of *H. hydrossis* in the sludge samples, with the corresponding bands of 118 base pairs (bps) by gel electrophoresis. These results were consistent with the primers' DNA design lengths (Table 1). Negative control was performed simultaneously to confirm the specific of the primers.

3.3.1. PCR

The slope of a standard curve was used to evaluate the gene copies of the real-time PCR. A real-time PCR standard curve was graphically represented as a semi-log regression line plot of Ct value vs. $\log_{10}$ of input nucleic acid.

Standard mixtures of known quantities of the plasmid were used to generate the standard curves relating the Ct values to the amounts of *H. hydrossis*. Fig. 4 showed the standard curve for *H. hydrossis*, ranging from 3.0×10^{-2} to 3.0×10^{-6} ng μL^{-1}, with the correlation coefficient of R^2 greater than 0.98, indicating that

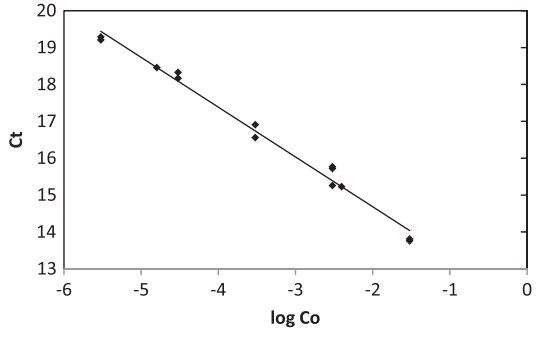


Fig. 4. Standard curve, used to quantify *H. hydrossis*. $C_t = -1.35$ log (C_0) +11.984, $r^2 = 0.988$, where C_t is the threshold cycle; C_0 is the initial concentration.

it was straightforward and reliable to determine the abundance of *H. hydrossis* in the activated sludge samples. The slope of the standard curve was −1.35.

Knowing the copy number and concentration of plasmid DNA, the precise number of molecules added to subsequent real-time PCR runs can be calculated, thus providing a standard for specific DNA quantification. Results are expressed as copy number per μL.

3.3.2. qPCR

After applying the appropriate amount of amplified DNA with the corresponding primers for targeting *H. hydrossis*, the copy number of the *H. hydrossis* genes can be qualified with the Ct values. qPCR analysis showed only one melting peak (Fig. 5). The specificity of the obtained PCR product was determined by cloning and sequencing. The reproducibility of qPCR was tested by comparing the curves and cycle thresholds of DNA samples. Replicate analysis of the same batch of sample showed an average standard deviation value of ±0.39, indicative of the reproducibility of results. Furthermore, it was also noted that the qPCR was a rapid and sensitive technique that can be used to identify and quantify the filament even at low abundances, which in turn facilitate the optimal functioning of activated sludge treatment process.

3.4. Correlation analysis

The total number of possible model parameters was nine. Any parameter exhibiting a Pearson's R (absolute value) of more than $1/N$ (known as "threshold coefficient of correlation") with *H. hydrossis* copies was selected as an independent model variable (where N = total number of parameters = 9, $1/N = 0.11$). The R value of Pearson's defines the percentage (%) of correlation that can be explained by a linear relationship [27]. Therefore, assuming that all possible independent parameters can equally contribute to the variation of *H. hydrossis* copies, the "threshold coefficient of correlation" signifies the minimum percentage of correlation (11%) that a parameter should exhibit with *H. hydrossis* copies for it to be identified (selected).

As the Pearson's R is useful for linear relationships and normal distribution only, the coefficient of rank correlation—Spearman's—was also studied. For example, if the Pearson's R (R_p) between *H. hydrossis* copies and a given parameter was zero or less than the threshold R, but the Spearman's R (R_s) was high, this would indicate a strong nonlinear relationship between the variables [27]. The converse of this would indicate a strong linear relationship. In addition, a situation where both the correlation statistics being more

Table 2
Correlation among the *H. hydrossis*, water parameters, and operational parameters of Macau Peninsula WWTP in July 2012–April 2013

	H. hydrossis (copies)	BOD_5	COD	SVI	MLSS	pH	NH_3-N	TN	TP	F/M
H. hydrossis (copies)	–	.071	.098	.335	−.161	−.063	.052	−.043	.059	.242
BOD_5	−.066	–	.787	−.173	−.212	−.161	.075	.020	−.269	.260
COD	−.112	.516	–	.067	−.187	−.178	−.088	−.068	−.346	.180
SVI	.721	−.163	−.122	–	−.167	−.082	.020	−.043	−.022	.243
MLSS	−.218	−.245	−.219	−.230	–	−.071	−.414	−.320	.051	−.877
pH	−.019	−.218	−.052	−.021	−.040	–	−.009	−.020	.139	.006
NH_3-N	.641	−.022	.016	.459	−.341	−.024	–	.296	.467	.348
TN	.386	−.116	−.076	.263	−.292	−.161	.549	–	.226	.339
TP	.232	−.247	−.157	.210	.145	.291	.459	.314	–	−.046
F/M	.277	.362	.039	.211	−.806	.007	.262	.210	−.095	–

Note: Coefficients above the diagonal (top right) are Spearman coefficients of rank correlation (R_s); coefficients below the diagonal (bottom left) are Pearson product-moment correlations (R_p). Assays are grouped based on clusters identified in Figs. 1 and 2. See text for a discussion of statistical significance.

than the minimum value (greater than 0.11) could indicate the need to consider both linear and nonlinear relationships between the parameters during the subsequent multivariate analyses. Pearson and Spearman correlation matrices were presented in Table 2.

From the results of Pearson and Spearman, SVI was anti-correlated with MLSS, due to the loss of activated sludge during bulking. Pearson results showed that *H. hydrossis* abundances were strongly correlated with SVI and NH_3-N, and weakly correlated with TP and F/M ratio relative to others, which suggested that the nutrients play an important role in the outgrowth of *H. hydrossis* during bulking (expressed in SVI). However, the relatively low correlation coefficients also implied that other factors besides nutrients may also involve in their growth.

Fig. 6(a) showed the SVI versus *H. hydrossis* 16S rRNA gene copies from 16 July 2012 to 26 April 2013. Measurements were done in triplicate for each qPCR run. It showed that the abundance of *H. hydrossis* fluctuated over the study period with the concentrations

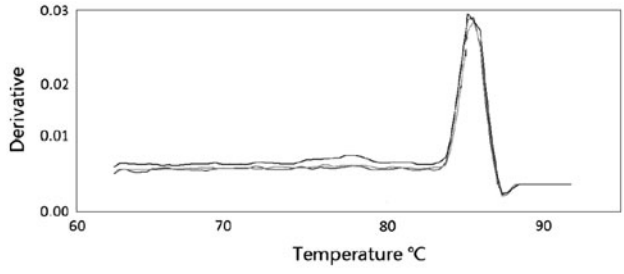
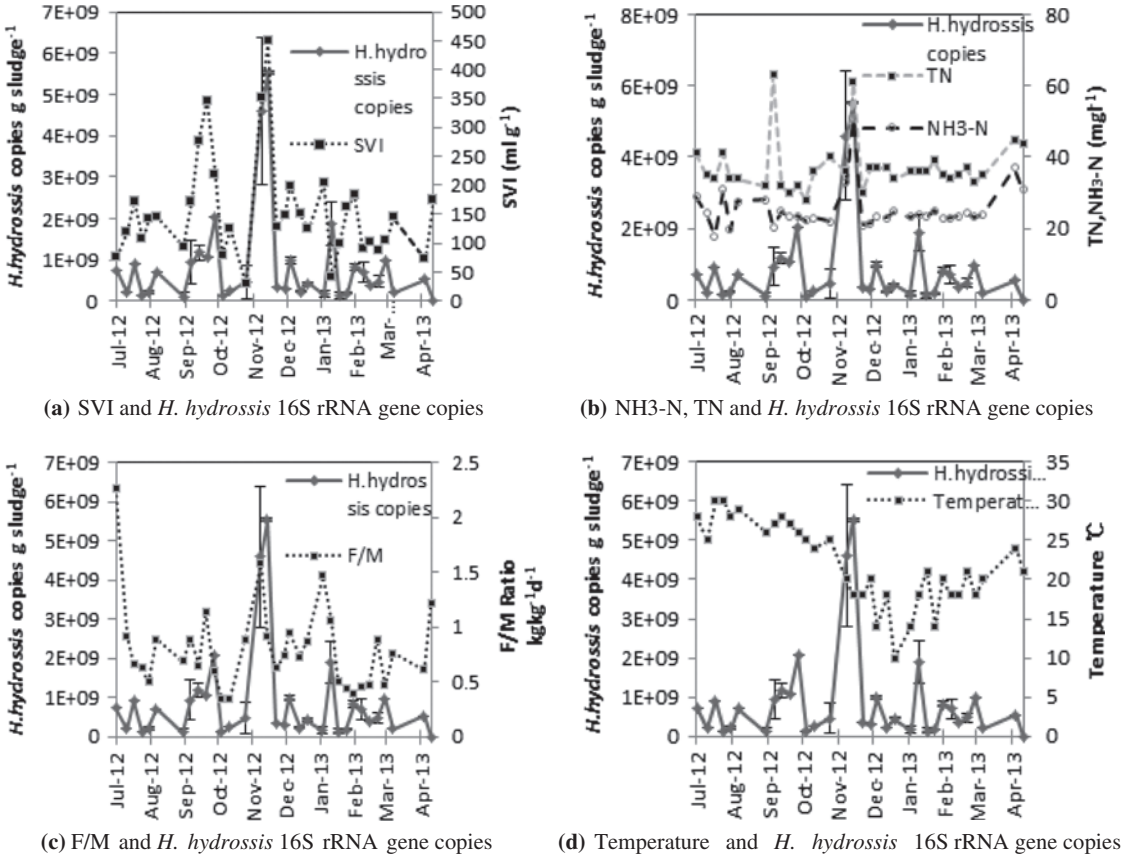


Fig. 5. A representative graph of the melting curve of the *H. hydrossis* 16s rRNA. Each sample was analyzed in duplicate. The specific product melts at 86℃.

from 1.07×10^8 to 5.25×10^9 gene copies g^{-1} sludge. The level of *H. hydrossis* gene copies in the present study that caused bulking was similar to that in the previous study [12], in which the dominant filaments of Type 021N reached the level of 10^9 gene copies. However, different from the previous study in 2008–2010 (Fig. 2) that most filamentous bulking happened in summer, the bulking in this study occurred in September–February, with the peak of 5.25×10^9 copies g^{-1} sludge and the corresponding SVI of 450 mL/g. Simultaneously, the SVI showed the similar pattern, with the values from 30 to 450 mL/g. Compared to the other water parameters, the coefficient of correlation between *H. hydrossis* copies and SVI got the greatest value ($R_p = 0.74$, $Rs = 0.34$). There was high correlation between *H. hydrossis* copies and SVI relative to other parameters. What's more, R_p was much more than R_s. This would indicate a linear relationship between the two factors. These results suggested that *H. hydrossis* was might the dominant filamentous bacteria causing the bulking.

The *H. hydrossis* concentrations and nitrogen source (NH_3-N and TN) were plotted in Fig. 6(b). It showed that NH_3-N occupied the relatively stable percentages of the TN in the wastewater, with the average of 68.9 mg/L. The NH_3-N concentration ranged around from 20 to 60 mg/L, which was consistent with the research outcomes from Kenny [28] stating that ammonia nitrogen within the sludge deposits can range between 20 and 500 mg/L. Through the means of diffusion, ammonia nitrogen which preserved in these sludge deposits transfer upwards into the aerobic layer [29]. As the quantity of sludge increase, more ammonia was released causing further nitrification.



(a) SVI and *H. hydrossis* 16S rRNA gene copies

(b) NH3-N, TN and *H. hydrossis* 16S rRNA gene copies

(c) F/M and *H. hydrossis* 16S rRNA gene copies

(d) Temperature and *H. hydrossis* 16S rRNA gene copies

Fig. 6. Water parameters and *H. hydrossis* 16S rRNA gene copies. 16S rRNA gene values are averages for three PCR runs.

NH_3-N and TN had the peak values of 61 and 51 mg/L, respectively, while dramatically decreased on 29 November 2013. The Spearman coefficient correlation (absolute value) for TN and NH_3-N was both less than the threshold 0.11 ($1/N$), even approximates to zero (TN: $R_s = -0.043$, NH_3-N: $R_s = 0.052$), might indicate that the linear relationship between *H. hydrossis,* and these two water parameters were too strong to presented by rank correlation. So, there was useless to select the spearman correlation to analysis. From the Pearson correct analysis results, high correlations occurred between the *H. hydrossis* concentrations and nitrogen source, with the correlation coefficients of 0.641 for NH_3-N and 0.386 for TN, indicating the *H. hydrossis* concentrations were ammonium nitrogen source dependence.

These results were consistent with the previous studies [30] that *H. hydrossis* favors inorganic nitrogen compound, particularly NH_3-N. And ammonia has been found by Williams and Unz [31] as the best nitrogen source for the filamentous bacteria in their study. However, it presented the *H. hydrossis* might can be influenced by the low concentration of NH_3-N below 60 mg/L, which did not correspond with the early study [32] that low ammonia concentration (10–1,000 mg/L) had no influence to *H. hydrossis.*

The F/M ratio was calculated and plotted in Fig. 6(c). The F/M ratios ranged from 0.35 to 1.58 kg BOD/kg MLSS/d, which was consistent with the research outcomes from Eikelboom [33] stating that *H. hydrossis* commonly occurs in activated sludge with the sludge load greater than 0.2 kg BOD/kg MLSS.d. However, it was contradictory to the previous study [34] that the presence of *H. hydrossis* was associated with low F/M ratio. Probably, other factors besides F/M ratio play another important role in inducing the outgrowth of *H. hydrossis* specie.

As the bulking was season dependence in 2008–2010 while showed different behavior in this study, further analysis in evaluating the effect of temperature on the dynamic change of *H. hydrossis* species was performed. The result was plotted in Fig. 6(d), indicating that the *H. hydrossis* abundance was temperature independence. Thus, temperature may be not the critical factor in controlling the filamentous bulking in Macau Peninsula WWTP. These results agreed with the

previous study [34] that *H. hydrossis* grows well at the temperatures of 8–30℃. Temperature has been reported to be the influencing factor on some filaments' growth. For example, an increase in *M. parvicella* population was observed during winter and spring season when the temperature was low (17–20℃), while a lower *M. parvicella* species was detected in the summer when the temperature was going high (> 20℃) [12]. It can be concluded that the sudden shift in the environmental temperature might be the control factor on the growth *M. parvicella*. However, the temperature control may not be effective method to suppress the growth of *H. hydrossis.*

In this study, TP concentration ranged around from 3.5 to 9.7 mg/L which was less than the minimum value distinct variation range of the earlier study that *H. hydrossis* preferred 50–200 mg/L of phosphorus concentration [35]. It was the possible reason that why the correlation coefficient between the concentration of TP and *H. hydrossis* was only 0.232 (R_p) in Table 2 compared to others. The phosphorus condition may did not favor the growth of *H. hydrossis*.

3.5. Multivariate analyses

It can be observed that there was a significant amount of multicollinearity among these parameters, judged by the correlation coefficients among them. The presence of high multicollinearity leads to inappropriateness, which should be solved by PCA. This can be achieved only to select a small number of parameters or components that would explain a high enough percentage (approximately 75% in this study) of the total variation in the parameters.

PCA was conducted to determine the correlations not only in one, but in the multivariate planes among different parameters [36]. The 10 variables including *H. hydrossis* abundances, water parameters, and process parameters were used in this study. The value of KMO was calculated as 0.6, the criteria value of applying the technique. The value of γ^2, calculated as 70.161 with *P*-value less than 0.0005 by Barlett's Test of Sphericity test, indicating that the PCA was applicable [37].

The scree test suggested there were four components (PCs) with the eigenvalues greater than 1, in which all the ten variables were included. PCA explained 75.21% of the variance from the total data in PC1-PC4. The biplot (Fig. 7) indicated that PC1 (30.76%) was most influenced by nitrogen sources and biological parameters. PC2 (21.92%) was mainly composed of biology oxygen demand and F/M ratio. PC3 (11.74%) and PC4 (10.79%) were defined as the pH and COD (Table 3).

The eigenvalues, variance proportion, and cumulative variance proportion were calculated and in Table 3, from which the eigenvectors can be obtained

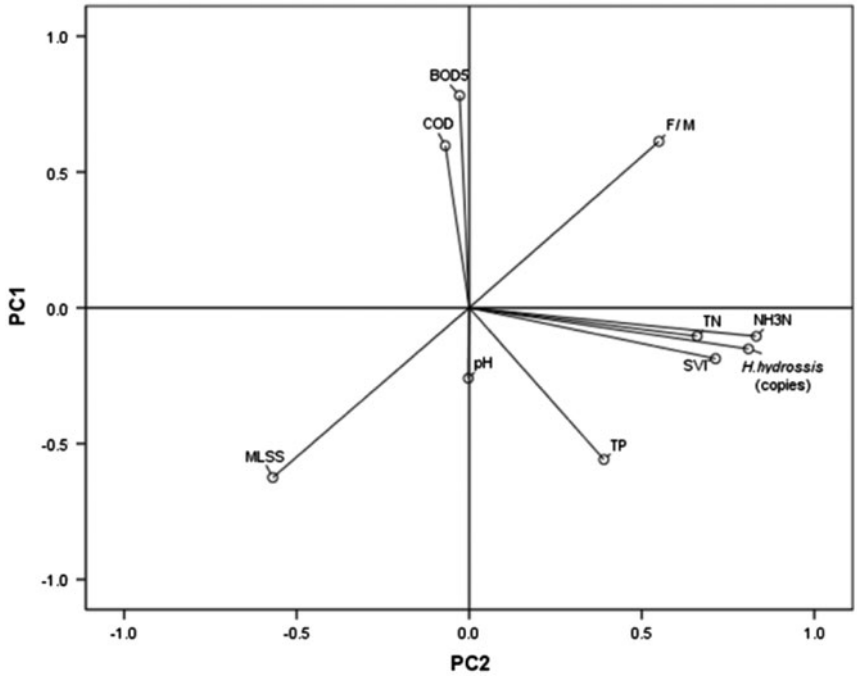


Fig. 7. The results of PCA in the ordination space of the first and second PCA axis (PC1 and PC2).

Table 3
Results of PCA ($k > 1$)

Variables	Component				Communalities
	PC1	PC2	PC3	PC4	
NH_3-N	.832	−.105	−.083	.297	.798
H. hydrossis (copies)	.809	−.151	−.171	−.073	.712
SVI	.714	−.187	−.137	−.201	.604
TN	.660	−.104	−.225	.139	.517
MLSS	−.569	−.625	−.323	.202	.860
FM	.550	.613	.318	−.305	.872
TP	.390	−.559	.234	.513	.782
COD	−.069	.597	−.047	.636	.767
BOD_5	−.028	.782	−.116	.325	.731
pH	−.003	−.259	.890	.141	.880

and presented in Table 4. There are totally ten PCs obtained by PCA.

The eigenvectors were used for this study. The parameters can be considered significant were the ones with the variance proportion greater than or equal to 0.50. It was indicated in Table 3 that MLSS less than 0.50 in PC1-PC4, and was thus classified as less important parameters in explaining the variance of the parameters, meaning that they may not be able to explain the filamentous bulking in the presence of *H. hydrossis*.

3.6. Regression analysis

In reference to the results of PCA, *H. hydrossis* gene copies number was regressed separately with SVI, NH_3-N, pH, BOD_5, COD, F/M, and TN. Table 5 showed the results for environmental variables regressed against *H. hydrossis* gene copies number using GLM. The interaction between copies and SVI, TN as well as NH_3-N was significant, with the p-value less than 0.0001 and 0.05, respectively. That consistent with the results of PCA, that nitrogen sources and SVI were the dominant explaining variables.

Table 4
Descriptive statistics of PCs

Component	Variance %	Cumulative %
PC1	30.758	30.758
PC2	21.92	52.678
PC3	11.743	64.421
PC4	10.791	75.212
PC5	8.964	84.176
PC6	5.712	89.888
PC7	3.689	93.576
PC8	3.433	97.009
PC9	1.999	99.008
PC10	0.992	100

The quadratic increases in the number of copies were observed with the increasing gradient of SVI, TN, and NH_3-N (Fig. 8(a)–(c)). Regression analysis GLM revealed a unimodal relationship between overall *H. hydrossis* gene copies number and three environmental variables. The copies number got a peak when SVI around 450 ml/g TSS (Fig. 8(a)), TN and NH_3 N around 60 mg/L (Fig. 8(b) and (c)). Positive linear correlation of *H. hydrossis* gene copies with SVI indicated their contribution to the bulking in the wastewater. All these three data sets revealed a unique "hump back-shaped" pattern of *H. hydrossis* gene copies with a nadir at about low level of the environmental gradient.

4. Discussion

Although the abundances and distributions of bacterial filamentous in activated sludge can be detected by high throughput sequencing accuracy, the database analysis which is the after-process needs a large abundance of compute devices. What's more, the operation cost for one typical sample can reach one thousand US dollars, which can be afforded by high level of institutions, instead of the small-scale laboratories. In addition, to our knowledge, no literature is available describing the long-term experiments using deep sequencing. Thus, real-time qPCR was applied for analyzing nearly whole year's sludge data in Macau Peninsula WWTP.

As mentioned before, 16S rRNA gene tree showing the phylogenetic affiliation of *H. hydrossis* within the phylum *Bacteroidetes*. Thin filamentous *Bacteroidetes* appear to be present in most WWTPs [38]. Based upon the complexity environmental conditions and

Table 5
Regression analysis results for environmental variables regressed against *H. hydrossis* gene copies number with GLM

Variables	Degree of freedom—residual	Residual (Log)	*F*-value	*P*-value
SVI	31	11.4	34.77	0.000002
NH_3-N	31	12.1	18.2	0.000178
TN	31	12.8	4.74	0.037305

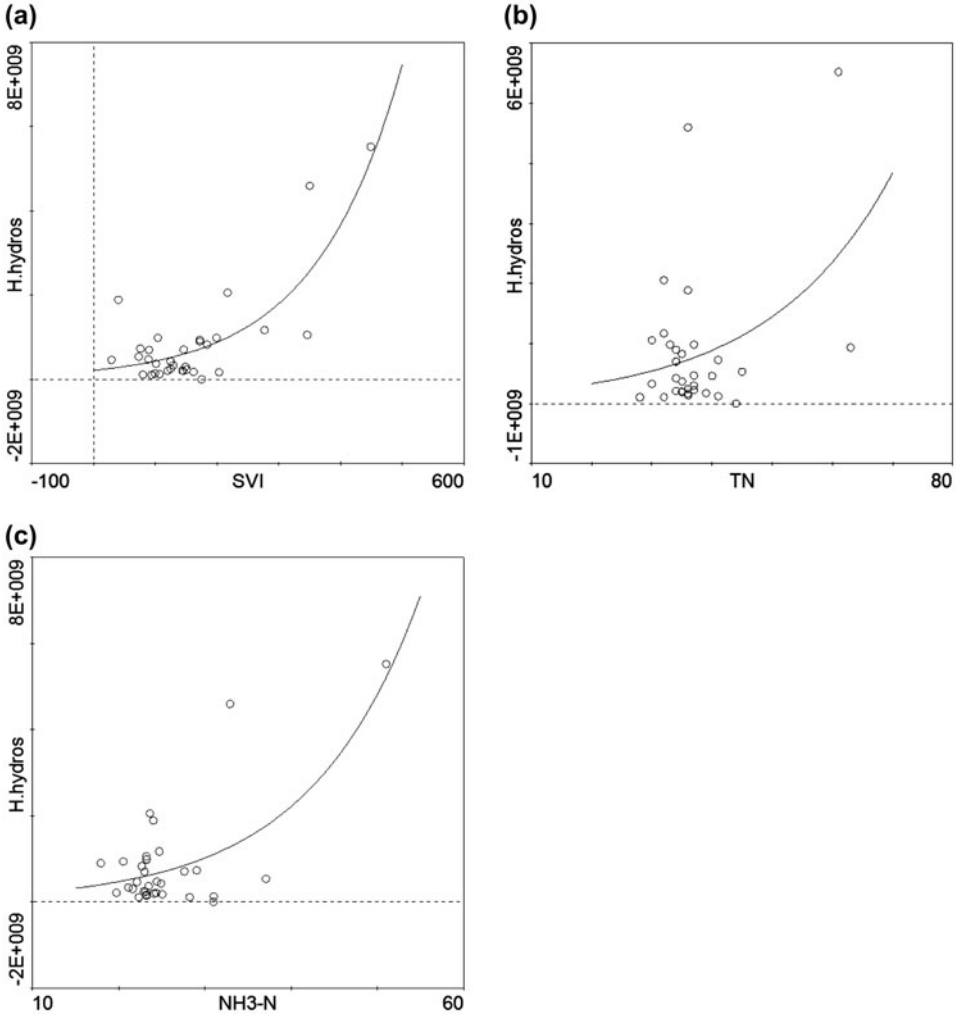


Fig. 8. Relationships between *H. hydrossis* gene copies number and environmental variables ($p < 0.005$).

conversion of dead microbial cells, it would be impossible to change one water parameter to control the filamentous bacteria growth in order to limit their abundance in WWTP. This study has given important information about the physiology and the growth of environment condition of *H. hydrossis* which was the dominant species in Macau WWTP, but has also shown that there was no obvious control measure. Describing the relation between this metabolic parameter and growth of filaments in a complex environment would result in a better understanding of the bulking phenomenon and the microbiology of activated sludge systems, as the direct causes of filamentous occurrence are impossible [39]. As both *H .hydrossis* gene copies and water parameters were quantified, the variation relationships between *H. hydrossis* abundances and water variables were described over time, respectively. Fig. 6 indicated that

measuring specific filamentous, such as *H. hydrossis*, over time can be used as a useful tool not only to predict the growth of this kind of filamentous bacterium, but also to evaluate the impacts of bulking reasons. Focusing on full-scale bulking incidents to confirm these results with a laboratory-scale reactor should be the future studies.

H. hydrossis was present in almost all WWTPs where USA, several European countries, South Africa, and Australia examined. It was considered as rarely responsible for bulking and foaming, which summarized by Jenkins et al. [3]. However, in this study, *H. hydrossis* 16S rRNA genes and SVI measurements were significantly correlated ($R_p = 0.72$), suggesting that the increase in *H. hydrossis* proliferation was responsible for the changes in the settling properties of the sludge. And it is now apparent that many filamentous species are specialized accommodator using a narrow range of growth requirements, while other filamentous species can, on the other hand, take up a large range of different growth requirements, e.g. the high ammonia concentrations (>2 g l^{-1}) inhibited the growth of type 1701 [40]. However, the other groups did not show significant growth differences at different ammonia concentrations. Phosphorus concentrations >0.01 g l^{-1} satisfied the phosphorus requirement for most of the groups, while type 021 N showed better growth at phosphorus concentrations ranging from 0.01 to 0.4 g l^{-1} [31]. So, it seems that many filamentous bacteria possess rather well-defined physiological and ecological traits, and if a better understanding of their ecophysiology is combined with knowledge on process conditions, better and more efficient control strategies may be developed. In rare cases where *H. hydrossis* cause problems, control is needed. This study has given important information about the physiology of the bacteria. Based upon its specialization on living environment of WWTP and abundance variation, it would be possible to using metabolic selection limit their abundance in WWTP [41].

The proliferation of *H. hydrossis* in WWTP could be attributed to a couple of main factors including nitrogen source, F/M ratio, dissolved oxygen, and sludge age. Controlling only one of such factor may not be help on limiting its growth. This study combining qPCR and PCA showed a strong influence of nitrogen source on the *H. hydrossis* that caused filamentous bulking expressed as SVI. The outcomes from the study can be used as the reference to control the *H. hydrossis* inducing bulking in Macau Peninsula WWTP. This also provides an opportunity to monitor the dynamic change of filaments in the plants and to change the appropriate strategy before filamentous bulking happens.

5. Conclusions

In this study, real-time qPCR was used to measure the weekly dynamic change of filamentous bacteria *H. hydrossis* of activated sludge samples in Macau Peninsula WWTP, from September 2012 to April 2013. Correction analysis, PCA, and GLM were applied for analyzing the relationships between *H. hydrossis* concentration and 9 water parameters. Our results showed that the techniques were successful for identifying and quantifying the *H. hydrossis*, with the highest concentration of around 5.5×10^9 copies g $sludge^{-1}$ on 30 November 2012. The results from correlation analysis suggested that ammonia nitrogen sources, SVI, and *H. hydrossis* have strong positive correlations relative to others, indicating that *H. hydrossis* played an important role in filamentous bulking of Macau Peninsula WWTP. In addition, PCA explained 75.21% of the variance from the total data in PC1-PC4, and GLM revealed a unimodal relationship between overall *H. hydrossis* gene copies number and three environmental variables. The quadratic increases in the number of copies were observed with the increasing gradient of SVI, TN, and NH_3-N. All these three data sets showed a unique "hump back-shaped" pattern of copies with a nadir at about medium level of the environmental gradient. The study indicated the *H. hydrossis* concentrations were ammonium nitrogen source dependence. The application of PCR and qPCR for the identification and quantification of filamentous bacteria from the activated sludge treatment plants will facilitate to monitor the dynamic change of the microbial population and thus assessing the effectiveness of the operations.

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