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Enumeration of protozoan ciliates in activated sludge: Determination of replicate number using probability

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ABSTRACT

A new approach to the enumeration of ciliate protozoa in activated sludge is described. A 25 μL sub-sample volume is optimal for routine analysis using a standard slide and 24 × 24 mm cover slip requiring between 20 and 40 min per sub-sample for full enumeration and identification of species. However, to achieve high probability (≥95%) of recovering all species large numbers of replicates are required (i.e. 23–47). To achieve high probabilities of recovery using less replicates it is necessary to neglect rare species with low densities (<0.5%); based on the assumption that they do not play a significant role in plant performance. The precise number of replicates required for different probabilities of recovering species is determined by conducting an initial pilot survey analysing a minimum of 8 replicates and using a probability equation to determine the optimum replicate number for that particular plant. Six replicate 25 μL sub-samples provided excellent species recovery (90–95% excluding up to 3 rare species), while analysing just two or three replicates, as commonly used in previous wastewater studies, only gave probabilities of 25 and 50% respectively for the same recovery. Ciliate analysis should be completed within 8 h of collection with significant changes in community structure occurring beyond this period.

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1. Introduction

Ciliate protozoa play a vital role in the activated sludge process by reducing the density of dispersed bacteria, including pathogens, by predation (Curds and Fey, 1969; Curds and Cockburn, 1970a); contributing to the flocculation process (Curds, 1963); and improving effluent quality by the removal of suspended and colloidal organic matter (Witthauer, 1980; Curds, 1982) thereby reducing both the BOD and suspended solids concentration of effluents (Curds and Fey, 1969; Curds and Cockburn, 1970a; Madoni, 2003).

Protozoa are sensitive to environmental change brought about by changes in influent quality or operating conditions, in particular sludge age, organic loading rate, f/m ratio and aeration intensity, that can lead to an alteration in their community structure which can subsequently affect performance (Curds and Cockburn, 1970b; Al-Shahwani and Horan, 1991; Esteban et al., 1991; Madoni, 1996). For this reason, ciliates are widely employed as indicators of activated sludge performance being associated with a wide range of physico-chemical and operational characteristics (Curds and Cockburn, 1970b; Poole, 1984; Al-Shahwani and Horan, 1991; Salvado et al., 1995). These indices are primarily based on ciliate diversity and abundance (Madoni, 1994; Burgess et al., 2002; Jiang and Shen, 2003).

Accurate enumeration of ciliate protozoa has become increasingly important both in research and operational management of activated sludge systems (Nicolau et al., 2001),

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with ciliate diversity now widely used to determine both system efficiency and effluent quality (Curds and Cockburn, 1970a,b; Madoni, 1994; Burgess et al., 2002). However, enumeration of ciliate populations has been the subject of little specific study. Researchers have used a variety of approaches for enumeration of ciliates, particularly in the volume of sample examined and the number of replicates, which varies widely. In their study of the use of ciliates as bio-indicators, Lee et al. (2004) took the mean of two replicate counts of 5 μL, while Lee and Oleszkiewicz (2003) used a single sub-sample of 20 μL while studying rotifer predation on nitrifiers. The mean of two 25 μL sub-samples has been used in a number of studies (Madoni, 1996,2000; Puigagut et al., 2007), while Zhou et al. (2006) used three 25 μL replicates of mixed liquor collected at the outlet end of the aeration tank. This method was also used by Salvado et al. (2001) examining the effects of shock loading of NaCl on protozoan in activated sludge and also by Martin-Cereceda et al. (1996) who carried out a comparative study of ciliate communities in ten activated sludge plants in Spain. Tyagi et al. (2008) used the same volume but a minimum of four replicates when comparing conventional and extended aeration plants. Ten replicates of 50 μL was employed by Chen et al. (2004) in their comparative study of the fauna at five activated sludge plants in Beijing. Other researchers have opted for much larger volumes for ciliate enumeration of 100 or 200 μL (Papadimitriou et al., 2007) using counting cells originally designed for algae and diatoms; while Burgess et al. (2002) used three replicates of 1000 μL with a deep-cell haemocytometer to measure protozoan diversity. The enumeration of flagellate protozoa is normally done in Fuchs Rosenthal chambers using a sub-sample volume of just 3.2 μL (Papadimitriou et al., 2007). Madoni (1984) collected samples from oligotrophic streams and activated sludge to compare replicate counts for a range of different sub-sample volumes (i.e. 3.2, 10, 25, 50 and 100 μL). From this he recommended using four 25 μL sub-sample replicates to achieve a total population estimate with a 25% error although the method does not consider the necessity of recovering all species present. Many researchers have adopted the 25 μL sub-sample volume, although few have used four or more replicates (Madoni, 1996; Martin-Cereceda et al., 1996; Madoni, 2000; Tyagi et al., 2008).

Ciliated protozoa can neither be kept for long periods nor successfully preserved, so microscopic examination is normally done *in vivo* as soon as possible (Madoni, 1984). In order to reduce the possibility of significant changes in the number of species and in abundance, various maximum time periods have been recommended in which the analysis should be completed. In most of the studies, samples have been analysed within 3 h (Al-Shahwani and Horan, 1991; Madoni et al., 1996; Madoni, 2000; Chen et al., 2004) or 5 h (Madoni, 1984, 1994), although Zhou et al. (2006) recommended that analysis should be done immediately or <3 h of collection. In contrast, Abraham et al. (1997), who checked for changes in the ciliate population structure during storage in flasks and under aeration over 48 h, found no significant change within 24 h.

However, in practice a short time period (<3 h), in which the analysis has to be done, can prove difficult especially when (i) sample collection is carried out at more than one location, (ii) where collection and analysis are done at different locations, or (iii) where the same person is responsible for both the microscopic analysis and same day physico-chemical analysis. Therefore, knowledge of the time during which no significant changes occur within the ciliate community ensures greatest flexibility of analysis without compromising accuracy.

The aim of this study was to establish a methodology to identify the optimal sub-sample volume for use with a standard microscope slide, the number of replicates for the examination of activated sludge protozoan community in order to recover as many species as possible, and to obtain a minimal error in population and species abundances. For this purpose three questions were asked: (i) What is the required number of replicates to recover maximum species from the sample? (ii) Is there a difference between a 20 and 25 μl sub-sample volume and (iii) is there an effect of sample storage on the protozoan community?

2. Material and methods

2.1. Samples of activated sludge

Activated sludge mixed liquor was collected directly from the aeration tanks of two different wastewater treatment plants. Ringsend Wastewater Treatment Plant (WWTP) is a large sequencing batch reactor (SBR) plant with denitrification, while Swords WWTP is an extended aeration biological nutrient removal (BNR) system. Both treat municipal wastewater from Dublin, Ireland. Collected samples were kept at ambient temperature and transported to the laboratory within one hour for immediate analysis where they were kept continuously aerated. The MLSS varied between 1800 and 2600 mg L^{-1} in the samples taken at different times.

2.2. Sub-sample size and replicates

Using mixed liquor collected from Ringsend WWTP the optimal sample size was determined. Mixed liquor was completely mixed by fully inverting the sample container three times to ensure homogeneity before sub-samples of 20 μL and 25 μL were taken using a gravimetrically calibrated automatic micropipette. A 25 μL sub-sample volume is most widely used by operators and researchers using a standard microscopic slide and glass cover slip. However, where there is a high density of flocs or protozoan, counting can take prolonged periods of time resulting in the material deteriorating. Therefore a smaller sub-sample volume of 20 μL was directly compared to the higher volume to assess if the same degree of accuracy could be achieved with reduced processing time. Eight replicates of each of the two test volumes were counted. One replicate of each sub-sample volume was prepared per slide in sequence so that any variation in enumeration due to temporal changes in either species diversity or abundance would be equally shared between the two selected volumes. After 25 μL had been identified as the optimal sub-sample volume counting of fresh samples from Swords WWTP and Ringsend WWTP was repeated using a greater number of replicates (i.e. 12 and 16 respectively), to retest the number of replicates required.

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