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A Comparison of Methods to Analyze Aquatic Heterotrophic Flagellates of Different Taxonomic Groups

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Heterotrophic flagellates contribute significantly to the matter flux in aquatic and terrestrial ecosystems. Still today their quantification and taxonomic classification bear several problems in field studies, though these methodological problems seem to be increasingly ignored in current ecological studies. Here we describe and test different methods, the live-counting technique, different fixation techniques, cultivation methods like the liquid aliquot method (LAM), and a molecular single cell survey called aliquot PCR (aPCR). All these methods have been tested either using aquatic field samples or cultures of freshwater and marine taxa. Each of the described methods has its advantages and disadvantages, which have to be considered in every single case. With the live-counting technique a detection of living cells up to morphospecies level is possible. Fixation of cells and staining methods are advantageous due to the possible long-term storage and observation of samples. Cultivation methods (LAM) offer the possibility of subsequent molecular analyses, and aPCR tools might complete the deficiency of LAM in terms of the missing detection of non-cultivable flagellates. In summary, we propose a combination of several investigation techniques reducing the gap between the different methodological problems.

Key words: Heterotrophic flagellates; method comparison; live-counting; fixation; liquid aliquot method; aliquot PCR.

Introduction

Heterotrophic flagellates (HF) are a very diverse and heterogeneous group of protists with a size range between 1 and 450 μm. They play an essential role in aquatic and terrestrial food webs as major consumers of bacterial biomass (Azam et al. 1983; Bonkowski 2004). However, quantitative data of HF were mostly restricted to the size group of "HNF" (heterotrophic nanoflagellates = size range

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of $\leq$15 μm) ignoring the high variability of flagellates with regard to morphology and dimension in aquatic habitats (Arndt et al. 2000). To resolve the role of HF in aquatic ecosystems, quantitative studies require accurate estimations of abundance and biomass and a reliable taxonomic resolution. The last issue is a prerequisite to adjust the activity of HF in different functional groups or guilds (Boenigk and Arndt 2002). However, this is especially challenging as the taxonomic affiliation is more difficult for most HF groups in comparison to ciliates. The latter are often considerably larger and they exhibit further useful morphological characters that can be studied in more detail (e.g. Foissner and Berger 1996).

During the last three decades, most methods were established based on fixed and stained samples investigated using epifluorescence microscopy (e.g. Caron 1983; Gifford and Caron 2000; Sherr et al. 1993). However, fixatives may significantly change the volume of HF (Chaput and Carrias 2002; Sonntag et al. 2000) and may lead to an important underestimation of flagellates due to species-specific treatment effects (e.g. Børsheim and Bratbak 1987; Choi and Stoecker 1989; Menden-Deuer et al. 2001). Though, live-counting has been considered as a suitable alternative method to analyze small sample droplets (e.g. Arndt and Mathes 1991; Gasol 1993; Massana and Güde 1991) it is still today not routinely applied. While a certain amount of expertise is needed to obtain a sufficient taxonomic resolution, the possible identification of functional guilds or even species groups or species is a great advantage of live-counting (Jeuck and Arndt 2013) and balances several of its disadvantages. Especially in ecological studies, the taxonomic classification of the dominant HF is required to deduce the ecological importance of HF.

In the present study, we compare different techniques to obtain best quality abundance, cell volume and taxonomic identification data of HNF in environmental samples including traditionally used quantification methods. Starting with evaluations of live-counting, we analyze fixation artefacts due to routinely used fixatives, search for additional alternatives such as the liquid aliquot method (LAM, Butler and Rogerson 1995) and the most probable number method (MPN, e.g. Baldock 1986; Ekelund et al. 2011; Sinclair and Ghiorse 1987), and consider an aliquot PCR (aPCR) technique. The latter one served as a special molecular tool of PCR without prior DNA extraction which was successfully used in bacteriology (Fode-Vaughan et al. 2001) and clinical research (e.g. Panaccio et al. 1993). The underlying advantages and disadvantages of every method (counting of fixed and living cells, cultivation, and molecular methods) were elucidated. Hence, we attempt to give a methodological survey of the here presented HF quantification techniques in addition to other techniques and to provide recommendations for reliable methods by considering studies of freshwater and marine habitats.

Results

Live-counting

Live-counting allowed the detection of the different morphological and behavioural features within diverse flagellate groups (Jeuck and Arndt 2013): the number of flagella (e.g. important for short second flagellum as present within *Spumella* sp.), specific modes of movement (especially relevant for e.g. free-living kathablepharids, bodonids, some euglenids), and the presence of specific cell structures like e.g. ejectisomes (e.g. cryptomonads). Long-term observations (1998-2015; Weitere and Arndt 2003 and unpubl. long-term data of H. Arndt) of HF by means of the live-counting technique (compare Weitere and Arndt 2003) in samples from the River Rhine at Cologne revealed all the main groups of HF: choanoflagellates, *Multicilia*, kinetoplastids, jakobids, euglenids, bicosoecids, chrysomonads, ciliophryids, dinoflagellates, cercomonads, glissomonads, cryomonads, cryptomonads, apusomonads, thaumatomonads, spironemids, kathablepharids, *Protista incertae sedis* (e.g. *Paramastix* sp., *Quadricilia* sp.; systematics according to Adl et al. 2012).

A comparison of live-counting results of different investigators revealed no significant differences ($p = 0.11$). The HF abundance estimates varied only slightly between the different investigators, irrespective of brackish or freshwater samples (Fig. 1). However, the live-counting error, the coefficient of variation, decreased significantly with the number of individuals counted per subsample. In case of more than 30 specimens counted in a well-mixed sample, the relative error of counting will be reduced to about 20% (Fig. 2).

While live-counting was found to be useful in eutrophic waters (freshwater and brackish waters), we were not able to use this method in oligotrophic environments (data not shown; see e.g. Arndt et al. 2003). Not only the abundance was often too low to be analyzed in droplets but also the prevalence of tiny forms smaller than 3 μm made it difficult to