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Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3–1000 m

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Abstract

Bioluminescent biomass in pure cultures and in 94 samples of shallow and deep Fennoscandian Shield groundwater was analysed using a commercial ATP assay, and the results were compared with microscopic counts and counts based on cultivation methods. The assay appeared robust and reliable and had a detection range that covered all samples analysed. The detection limit in groundwater was determined to 2×10^3 cells ml^{-1}. ATP concentrations were found to correlate with the microscopic counts and with the volume and metabolic status of the investigated pure culture and groundwater cells. The results suggested that bacterial populations in deep groundwater vary significantly in size, and that metabolic activity is a function of prevailing environmental conditions. In cases in which analysis of the total and viable number of cells produced very low numbers, suggesting that the detected cells were of low viability, ATP analysis of the ratio of ATP to the total number of cells was able to verify such an interpretation of the obtained data.

Keywords: ATP; Biomass; Biovolume; Groundwater; Subsurface; Microbial

1. Introduction

The subsurface biosphere accounts for a significant amount of the overall biomass on earth (Whitman et al., 1998). The microbial biomass in granitic rock aquifers of the Fennoscandian Shield has been analysed in terms of total and viable numbers for almost two decades (Pedersen, 2001). Total numbers ranged from 10^3 to 10^6 cells ml^{-1}, while viable numbers ranged from 1 to 10^5 cells ml^{-1}. Between 0.00084 and 14.8% of the total numbers were cultivated and detected using most probable number (MPN) methods (Haveman and Pedersen, 2002). Although low viable numbers were detected relative to the total numbers observed, *in vitro* radiographic and radiotracer estimates suggested that the absolute majority of the total cells observed using microscopy were viable (Pedersen and Ekendahl, 1990, 1992a,b). Consequently, there was a significant gap between estimates of potentially viable total numbers and evidently viable cultivable numbers. Hence, a robust, sound, and relatively simple method to estimate the total amount of viable biomass in groundwater was sought.

As microbial mass itself is difficult to measure directly, various proxies are commonly applied, including the analysis of cell numbers, *e.g.* analysis of total cell numbers using epifluorescent microscopy (Hobbie et al., 1977) and various types of viable number counts obtained with solid or liquid media. Analysis of specific cell components is possible when biomass and its particular constituents occur in stable ratios. Biomass can be estimated relative to the amounts of DNA (Tranvik, 1997), membrane lipids (White et al., 1979), proteins (Bradford, 1976), and adenosine triphosphate (ATP) (Lundin et al., 1986). ATP estimation has the advantage of estimating only the viable, living biomass (Lundin et al., 1986). The firefly luciferase bioluminescence method was first proposed for biomass estimates in seawater (Holm-Hansen and Booth, 1966) and has since been used to estimate viable biomass in a wide range of applications such as algae (Holm-Hansen, 1970), coastal sediments (Oulahal-Lagsir et al., 2000) and diary products (Koster and Meyer-Reil, 2001). Several modifications of the method have been developed, such as enhanced bioluminescence with diethylaminoethyl-dextran (Ishida et al.,

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2002), extraction of ATP using benzalkonium chloride with a mutant luciferase resistant to benzalkonium chloride (Hattori et al., 2003), and recombinant luciferase that allows the preparation of ATP reagents with high luciferase activity (Lundin, 2000).

The volume of discrete microbial cells and their ATP contents have been found to vary by several orders of magnitude under different conditions. This has been demonstrated in batch and chemostatic cultures of marine bacteria in artificial seawater (Hamilton and Holm-Hansen, 1967), batch cultures of a marine isolate and *Enterobacter aerogenes* grown in artificial seawater (Bancroft et al., 1976), batch cultures of *Streptococcus faecalis* grown in, for example, peptone, yeast extract, glucose, and sodium citrate (Forrest, 1965), algal batch cultures grown under different nutrient and light conditions (Holm-Hansen, 1970), and cells under starvation in a biomass recycle reactor (Konopka et al., 2002); however, granitic groundwater has not been examined. Therefore, the applicability of the ATP method and the relationships between total and viable numbers, biovolume, and ATP of microorganisms in shallow and deep groundwater were investigated in this work.

The firefly bioluminescence assay developed by Lundin (2000) has a detection limit of 5–50 cells per ml^{-1} and delivers reproducible results within minutes. Biomass of microbial populations in groundwater from 3 to approximately 1000 m depth in overburden soils and hard rock aquifers was analysed using this ATP assay and compared to total and viable numbers biomass estimates. Similar comparisons were performed on pure cultures in the laboratory. The ATP content per cell and per cell volume in samples from different groundwater sites were calculated and compared with the amount of ATP per cell and per cell volume in samples from pure cultures. The relationships between the amount of ATP, the total and viable numbers, and the volume of cells were analysed. The relationships between ATP concentrations, cell numbers, and cell volumes in the analysed groundwater were calculated, and the usefulness of various ratios for evaluating microbial activity were explored.

2. Material and methods

2.1. ATP analysis

The ATP biomass kit HS for total ATP in living cells was used (No. 266-311, BioThema AB, Handen, Sweden). This analysis kit was developed based on the results of Lundin et al. (1986) and Lundin (2000). Sterile and PCR clean epTIPS with filters (Eppendorf, GTF, Göteborg, Sweden) were used in transferring all solutions and samples to exclude ATP contamination of pipettes and solutions. Light may cause delayed fluorescence of materials and solutions, so all procedures described below were preformed in a dark room and all plastic material, solutions, and pipettes were stored in the dark. A new 4.0-ml, 12-mm-diameter polypropylene tube (No. 68.752, Sarstedt AB, Landskrona, Sweden) was filled with 400 µl of the ATP kit reagent HS (BioThema, Handen, Sweden) and inserted into an FB12 tube luminometer (Sirius Berthold, Pforzheim, Germany). The quick measurement FB12/Sirius software, version 1.4 (Berthold Detection Systems, Pforzheim, Germany) was used to calculate light emission as relative light units per second (RLU s^{-1}). Light emission was measured for three 5-s intervals with a 5-s delay before each interval, and the average of the three readings was registered as a measurement. The background light emission (I_{bkg}) from the HS reactant and the tube was monitored and allowed to decrease to a value below 50 RLU s^{-1} prior to registration of a measurement. ATP was extracted from 100-µl aliquots of sample within 1 h of collection by mixing for 5 s with 100 µl of B/S extractant from the ATP kit in a separate 4.0-ml polypropylene tube. Immediately after mixing, 100 µl of the obtained ATP extract mixture was added to the HS reactant tube in the FB12 tube luminometer, and the sample light emission (I_{smp}) was measured. Subsequently, a volume of 10 µl of an internal ATP standard was added to the reactant tube, and the standard light emission (I_{std}) was measured. The concentration of the ATP standard was initially 10^{-8} M; it was increased 10-fold to 10^{-7} M by the end of 2004 by the producer (BioThema AB, Handen, Sweden). Samples with ATP concentrations close to or higher than that of the ATP standard were diluted with B/S extractant to a concentration of approximately 1/10 that of the ATP standard. Mixtures of HS reactant and B/S extractant were measured at regular intervals to control for possible ATP contamination. Values of 500±400 amol ATP ml^{-1} ($n=10$) were obtained with clean solutions, while solutions displaying values above 500 amol ATP ml^{-1} were disposed of.

The ATP concentration of the analysed samples was calculated as follows:

$$\text{amol ATP ml}^{-1} = (I_{smp} - I_{bkg})/((I_{smp+std} - I_{bkg}) - (I_{smp} - I_{bkg})) \times 10^9 / \text{ sample volume} \quad (1)$$

where I represents the light intensity measured as relative light units s^{-1}, smp represents sample, bkg represents the background value of the HS reagent, and std represents standard (all referring to a 10^{-7} M ATP standard).

2.2. Determination of total number of cells

The total number of cells (TNC) was determined using the acridine orange direct count (AODC) method of Hobbie et al. (1977), as modified by Pedersen and Ekendahl (1990). All solutions used were filtered through sterilized 32-mm-diameter, 0.2-µm pore size Filtropur S syringe filters (Sarstedt, Nümbrecht, Germany). Stainless steel analytical filter holders, 13 mm (No. XX3001240, Millipore, Billerica MA, USA), were rinsed with sterile filtered 2% HCl (to remove possible iron oxide percipitates) and double distilled water (DDW) for groundwater samples and with sterile filtered DDW for laboratory samples prior to filtration. Samples of 1 ml were suction filtered (−20 kPa) onto 0.22-µm pore size Sudan black-stained polycarbonate filters, 13 mm in diameter (Osmonics, Minnetonka, MN, USA). The filtered cells were stained for 5 min with 200 µl of an acridine orange (AO) solution (SigmaAldrich, Stockholm, Sweden). The AO solution was