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NEW METHOD USING GROWTH DYNAMICS TO QUANTIFY MICROBIAL CONTAMINATION OF KAOLINITE SLURRIES

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Abstract—The early and sensitive detection of microbial contamination of kaolinite slurries is needed for timely treatment to prevent spoilage. The sensitivity, reproducibility, and time required by current methods, such as the dip-slide method, do not meet this challenge. A more sensitive, reproducible, and efficient method is required. The objective of the present study was to develop and validate such a method. The new method is based on the measured growth kinetics of indigenous kaolinite-slurry microorganisms. The microorganisms from kaolinite slurries with different contamination levels were eluted and quantified as colony-forming units (CFUs). Known quantities of *E. coli* (ATCC 11775) were inoculated into sterilized kaolinite slurries to relate kaolinite-slurry CFUs to true microbial concentrations. The inoculated slurries were subsequently incubated, re-extracted, and microbial concentrations quantified. The ratio of the known inoculated *E. coli* concentration to the measured concentration was expressed as the recovery efficiency coefficient. Indigenous microbial communities were serially diluted, incubated, and the growth kinetics measured and related to CFUs. Using the new method, greater optical densities (OD) and visible microbial growth were measured for greater dilutions of kaolinite slurries with large microbial-cell concentrations. Growth conditions were optimized to maximize the correlation between contamination level, microbial growth kinetics, and OD value. A Standard Bacterial Unit (SBU) scale with five levels of microbial contamination was designed for kaolinite slurries using the experimental results. The SBU scale was validated using a blind test of 50 unknown slurry samples with various contamination levels provided by the Imerys Company. The validation tests revealed that the new method using the SBU scale was more time efficient, sensitive, and reproducible than the dip-slide method.

Key Words—Contamination, Dip-slide Method, SBU Method, Kaolinite, Microbial Growth Kinetics.

INTRODUCTION

Kaolinite (white or near-white clay mineral), primarily in the form of slurries, is used in the production of paper, ceramics, medicines, and paints (Murray, 1991, 2000; Bundy, 1990). Georgia kaolinite, in large part, stems from the weathering of muscovite and altered feldspars of a unique and intricate chemical structure with SiO_2 and Al_2O_3 being the most prevalent compounds (Murray, 1991; Nyakairu *et al.*, 2001; van Olphen and Fripiat, 1979). Kaolinite 1:1 structural units consist of a tetrahedral silica sheet joined to an octahedral alumina sheet with charges along the structure edges and faces that provide absorptive and adsorptive properties (Murray, 1991; Herrington *et al.*, 1992). The absorptive and adsorptive traits of kaolinite along with the color and nearly universal abundance make it ideal for industrial applications (Murray, 1991). Kaolinite chemistry, however, also makes an accommodating environment for bacterial populations.

The outer wall of bacteria carries a predominantly negative charge that enables interaction with charged surfaces through van der Waals or electrostatic attraction (van Loosdrecht *et al.*, 1987) that may be strengthened further by lipopolysaccharide interactions (Allison and Sutherland, 1986; Chenu, 1993). This charge interaction allows bacterial species to bind to bacteria of the same species, to other bacteria, or to particles. Microorganisms adsorb strongly to kaolinite particles and act as contaminants that adversely affect kaolinite slurries. Microbial contamination effects include dark blemishes, a fetid smell, and an altered viscosity which render the product problematic (Drew *et al.*, 1996). Spoiled slurries could potentially be reclaimed on-site at the mill and made fit for use by treatment with oxidants, but this process is time consuming and involves substantial cost.

Whether contamination of kaolinite slurry is caused by indigenous or by introduced microorganisms is unknown. Introduction of microorganisms may occur during kaolinite mining, processing, amendment with dispersants, and transport/storage (Guthrie and Ashworth, 2002). The dip-slide method is commonly used as a tool for detecting microbial contamination in slurries. This method suffers from low sensitivity and reproducibility (Rabenstein *et al*., 2009). For timely detection of microbial contamination, a more sensitive and reproducible method than the dip-slide method currently employed by industry is required.

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Methods based on DNA or ATP analysis are not suitable for quantifying microbial contamination in kaolinite-dominated environments because both DNA (Paget *et al.*, 1992; Ogram *et al.*, 1994; Lorenz and Wackernagel, 1994; Frostegård *et al.*, 1999) and ATP (Jenkinson and Oades, 1979; Graf and Lagaly, 1980) adsorb readily to kaolinite and other clay particles.

The aim of the present study was to develop a sensitive, accurate, and efficient method for determining microbial contamination in kaolinite slurries. The growth dynamics of microorganisms isolated from kaolinite slurries were hypothesized to reflect the level of microbial contamination in kaolinite slurries. Anaerobic microorganisms that might produce the dark blemishes and the fetid smell in kaolinite products develop slowly (Sennett and Brown, 1991; Shelobolina *et al*., 2005). Hence, aerobic and facultative anaerobic microorganisms were selected for use as a measure to reflect the general contamination level. In previous research, an elution protocol for effective isolation of microorganisms from various environments was developed (Barkovskii and Fukui, 2004). Kaolinite microorganisms that are eluted using this protocol were expected to grow in a nutritional medium. Growth dynamics postulates that particular microbial cell numbers are required to initiate visible microbial growth. Sequential dilutions of kaolinite microorganisms were, therefore, expected to reveal a dilution in which microbial growth is imperceptible due to insufficient microbial cell numbers. Kaolinite slurries with greater microbial cell numbers should produce visible growth at greater dilutions. Visible growth can be detected and recorded as absorbance or optical density (OD) units. The resulting microbial density is proportional to the number of inoculated cells and can be represented by two numbers: (1) the greatest dilution with visible microbial growth; and (2) the maximum OD measured at this dilution after a suitable incubation time.

MATERIALS AND METHODS

Elution and incubation of kaolinite-slurry microbial community

Contour^{TM}100 (trademark owned by Imerys, C100), a fine delaminated hydrous kaolin pigment slurry for paper applications produced from Georgia kaolinite (pH 6.50−6.80 and 67.8−68.2% solids), was selected for use in the present study because of the occasional spoilage problems with this product that have been reported at the processing plant. The slurry was obtained directly from the plant tanks. Microbial communities in the kaolinite slurries were eluted with a modified (Barkovskii and Fukui, 2004) buffer solution (potassium phosphate buffer, pH 6.8) amended with 0.01% polyoxyethylene sorbitan monooleate (Tween 80, Sigma Aldrich, St. Louis, Missouri, USA), which is referred to as KPT80. In this solution, the buffer provided pH equal to that of slurries, and the surfactant, Tween 80, weakened the bonds between kaolinite particles and microorganisms (Scheraga *et al.*, 1979; Barkovskii and Fukui, 2004). Kaolinite slurry samples (20 mL) were added to 50 mL screw-cap centrifuge tubes (Fisher Scientific) and eluted with 30 mL of KPT80 buffer solution. Centrifuge tubes with samples were hand shaken vigorously for 2 min followed by 5 min of ultrasonic treatment (Gem-Sonic, Whitman, Massachusetts, USA). Samples were centrifuged for 30 min at 22ºC (Thermo Electron Corporation, IEC Multi RS Refrigerated Centrifuge) using a swing-bucket rotor at 1,100 relative centrifugal force (RCF). The supernatant was diluted serially down to $1{:}10^{-9}$ in sterile Luria-Bertani (LB) broth media in a sterilized laminar-flow hood. Before incubation of serial dilutions, aliquots of each dilution were collected, plated aseptically in triplicate on LB agar plates (of 100 µL/plate), and spread with an L-shaped plate spreader. The plates were subsequently incubated at 37ºC, CFUs were counted after 24 and 48 h of incubation, and microbial abundances per 1 mL of slurry were calculated and corrected for recovery efficiency as described below.

Serial dilutions were sealed with foam inserts and incubated at 37ºC for 16−28 h with agitation at 130 rpm, or without agitation (Fine PCR Hybridization Incubator Convi-SV12DX, Daigger, Seoul, South Korea), and at varying surface-to-volume ratios. The OD of the serial dilutions was recorded at 560 nm before and after incubations (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific, Madison, Wisconsin, USA). Correlations between OD and CFUs were established, and the lower and upper microbial contamination thresholds were determined.

Recovery efficiency

Kaolinite-slurry samples were sterilized by autoclaving (121ºC, 30 min), inoculated with known quantities of *E. coli* (purchased from American Type Culture Collection (ATCC), reference number 11775), and incubated at 4ºC for 24 h to allow adsorption to kaolinite particles, but to prevent microbial growth. The *E. coli* cells were eluted from the samples with the elution protocol using the KPT80 buffer, and plated on eosin methylene blue (EMB) plates for selective exposure of *E. coli* colonies. The plates were incubated at 37ºC for 24 h and counted. The recovery efficiency was calculated by comparing the microbial counts obtained for *E. coli* cells before and after exposure to kaolinite slurries.

Optimization of parameters and designing the SBU scale

Dynamic and static incubation trials were run to evaluate the microbial growth patterns using various incubation times and surface-to-volume ratios. A desired time period of 16 h was specified by Imerys for collection of microbial quality-control data. Indigenous and unintentionally introduced slurry microorganisms were eluted with the elution protocol using the KPT80

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buffer, serially diluted up to 10^{-9}, and incubated in 20 mL test tubes and 250 mL Erlenmeyer flasks with 9 mL and 45 mL of LB broth media volumes, respectively, at 37ºC for 16, 24, and 48 h to represent equally faster- and slower-growing microorganisms. The CFUs were counted, compared, and the microbial concentrations in each dilution were calculated. The resulting protocol (described fully in the 'Results and Discussion' section) was named Protocol 1 (P1) and used in all the subsequent experiments. A microbial propagation technique was used to correlate the dilution of microbial load of the kaolinite slurry with visible microbial growth and with the dilution OD reading. Six slurry samples with undetectable contamination were propagated for 24–144 h at 37ºC and samples were removed sequentially every 24 h. These samples were treated with P1, and correlations between microbial abundance and growth dynamics were established. Optimized parameters were used in designing the Standard Bacterial Unit (SBU) scale. Two SBU numbers on the SBU scale are used to describe the dilution and OD value of each microbial load. The first number indicates the greatest dilution with visible microbial growth (*e.g.* a dilution of 1:1000 is designated '3' on the SBU scale). The maximum OD value of the dilution after a suitable incubation time is indicated by the second number (*e.g.* OD value of 1.5 is designated 15 on the SBU scale).

Method validation

The SBU method developed was tested for sensitivity, efficiency, and reproducibility and compared to the dip-slide method. Fifty kaolinite-slurry samples of unknown contamination levels were prepared, labeled randomly, and delivered to Georgia College and State University (GCSU) by the Imerys Company. An identical sample set was kept by Imerys and tested using the dip-slide method. The GCSU laboratory results were reported to Imerys and compared to the dip-slide method data.

RESULTS AND DISCUSSION

Method development

The modified Barkovskii and Fukui (2004) elution protocol successfully eluted microorganisms from two similarly contaminated samples (Figure 1a,b). Successful elution of microorganisms was expected because the original Barkovskii and Fukui (2004) protocol was designed for the elution of microorganisms

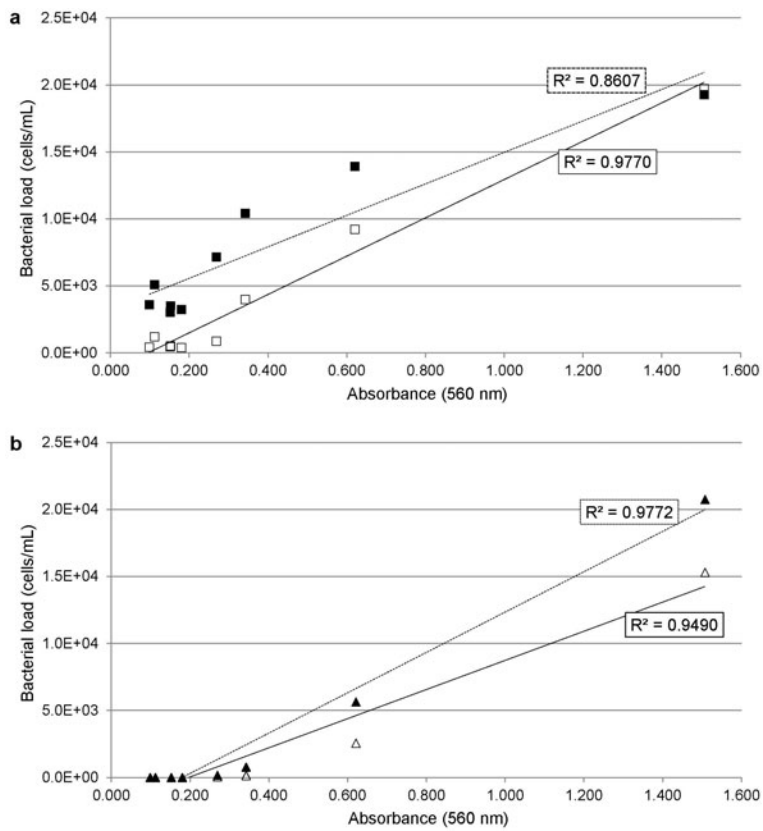


Figure 1. Microbial loadings of two independently collected kaolinite slurry samples (a and b) as reflected by OD values after 16 h of incubation of eluted microorganisms in LB broth, and by CFU counts after 24 h (open symbols) and 48 h (solid symbols) of propagation of the eluted microorganisms on LB agar. The CFU standard deviations did not exceed 10% for each data point.

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associated with clayey soil particles. The protocol is efficient in various matrices and has been applied successfully to the elution of microorganisms from rhizospheric soils (Zhang *et al*., 2011), feathers (Kulkarni, 2005), and textiles (Rabuza *et al*., 2012). Correlations between absorbance values (OD readings) and microbial counts were satisfactory at high contamination levels, but correlations between the low OD readings and microbial counts at low contamination levels were unsatisfactory. Limitations imposed by the incubation conditions probably caused the discrepancies above and were subsequently evaluated and optimized.

The agents and metabolic processes that cause kaolinite-slurry spoilage are virtually unknown. An association between *Pseudomonas spp*. and *Acinetobacter spp*. and slurry rheology was suggested by Papp *et al*. (2012); an association between sulfate-reducing microorganisms and dark blemishes was inferred by Osipov and Turova (1997). Limited oxygen affects negatively the growth kinetics of the aerobic and facultative anaerobic microorganisms chosen to reflect kaolinite-slurry contamination levels. Agitation is used widely to improve oxygen mass transfer in incubation media. Agitation during test-tube incubation impacted positively on microbial growth dynamics and improved resolution at lower levels, although this improvement was insufficient for the intended purpose, especially due to inconsistency between replicates proven by the error bars (Figure 2). The surface-to-volume ratios in incubation containers were increased to further improve oxygen and nutrient mass transfer. The 20 mL test tubes had a surface-to-volume ratio of 1:5 cm^2/cm^3. Use of 250 mL Erlenmeyer flasks instead of test tubes increased the ratio to 1:1 cm^2/cm^3. This enhanced the OD values and reading accuracy significantly during incubation and eliminated potential errors in interpretation (Figure 2). Incubation time was also increased to improve measurement accuracy. The previous 16 h of incubation time was insufficient for the eluted microbial communities to reach the stationary phase and large deviations in logarithmic-phase microbial community growth dynamics were noted (Figure 2). A 24 h incubation time was chosen during development of the SBU method. After 24 h of incubation, the early stationary phase with very minor OD reading deviations was reached. No growth was observed in microbial communities eluted from uncontaminated kaolinite samples (Figure 3).

The recovery efficiency of the eluted microorganisms was evaluated using the modified Barkovskii and Fukui (2004) elution method followed by microbial plating and counts as described in the 'Materials and Methods' section above. The recovery of microbial cells, based on the *E. coli* cells introduced, revealed a 1.91±0.12% efficiency that was rounded to 2.00%, divided by 100, and expressed as a recovery coefficient of 0.02 for all subsequent calculations of microbial abundance. The greatest abundance of microorganisms was 10^5 cells/mL after correction for the recovery efficiency and this value is consistent with the data of Osipov and Turova (1997). The recovery efficiency of this protocol seems small but is considerably better than values reported previously, *e.g.* 9% of all soil bacteria were extracted from a sandy soil (48% sand, 35% silt, 17% clay) by Maron *et al*. (2006), but only 0.5% of the cells from a clayey, acidic, tropical forest soil (24% sand, 6% silt, 70% clay). The resulting protcol, consisting of (1) the modified microbial elution method using the KPT80 buffer, (2) incubation of the eluted microorganisms in 250 mL Erlenmeyer flasks for 24 h at 37ºC and 130 rpm, (3) microbial plating, and (4) colony counts corrected for the recovery coefficient, was given the name Protocol 1 (P1) and was used in all the subsequent experiments.

Development of the SBU scale

Protocol 1 was tested on slurries with a range of contamination levels. The experiment consisted of six

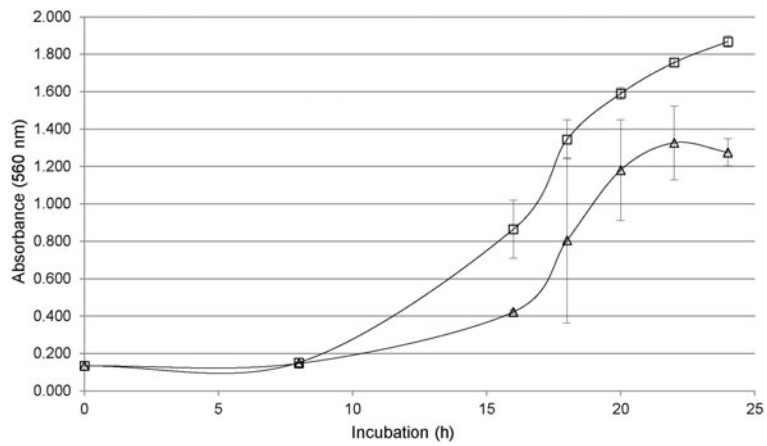


Figure 2. Kaolinite microorganism growth patterns during agitation in 20 mL test tubes (open triangles) and 250 mL Erlenmeyer flasks (open squares).

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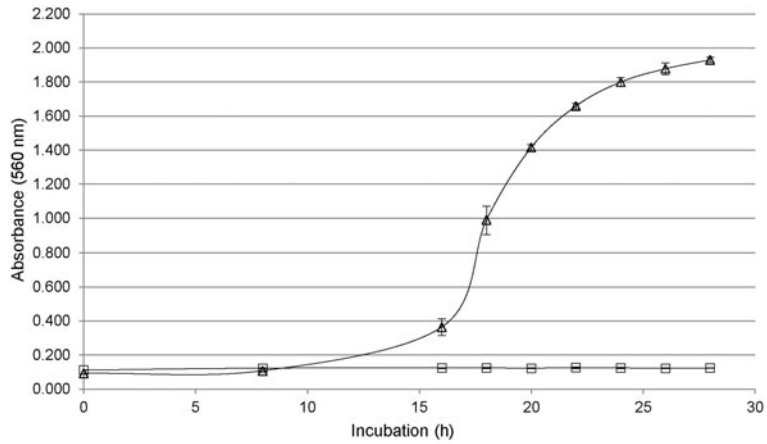


Figure 3. Growth patterns of microorganisms eluted from contaminated (open triangle) and sterilized (open square) kaolinite slurries during 28 h of incubation. The standard deviation bars for sterilized kaolinite-slurry elutes were below the detection limit.

slurry samples from the same source with no detectable microbes. Each sample was propagated, eluted, diluted to 10^{-5}, incubated, and quantified as described above. Before propagation, no microbes were detected by CFU counts. No microbial growth was found in dilutions of 10^{-3} for elutes derived from slurries propagated for 24 to 44 h after incubation (Figure 4). The greatest microbial abundance of 6×10^5 cells/mL was measured in 10^{-1} to 10^{-3} dilutions of a 144 h propagation and was detected by microbial counts and visually by OD readings. The next highest contamination level of $3-6 \times 10^5$ was detected in $10^{-1}-10^{-3}$ dilutions. A microbial contamination level of $1-3 \times 10^5$ for a 96 h propagation was only detected in the 10^{-2} and 10^{-1} dilutions. A microbial contamination level of 1×10^3 to 5×10^4 for 24–72 h of propagation was detected visually, by OD readings, and from microbial counts, but only in the 10^{-1} dilution. Microbial contamination levels of $<1 \times 10^3$ cells/mL were not detected by the method. The testing performed demonstrated that P1 reflects reliably a wide range of microbial concentrations in kaolinite slurries and can be used to detect the microbial contamination level.

The Standard Bacterial Units (SBU) scale was designed using the greatest dilution with visible microbial growth and the OD readings in this dilution as measured values. For each contamination level, the SBU values were arranged into two numbers (*e.g.* 3:15 SBU). The first SBU number (3 in this case) is the greatest dilution with visible microbial growth and the second SBU number (15 in this case) represents the OD reading multiplied by 10 (*e.g.* OD = 1.5 as 15) (Table 1). The SBU scale can be compared to a clock where the dilution is the hour hand and OD is the minute hand. Taken together, the dilution and OD readings accurately reflect kaolinite-slurry contamination levels.

The resulting method using P1 (except microbial plating and colony counts) as a procedure and the SBU chart for the determination of microbial contamination levels was named the Standard Bacterial Units (SBU) method. This method for determining microbial contamination of kaolinite slurries consisted of the following procedures and elements: (1) transfer 20 mL of kaolinite slurry into a 50 mL centrifuge tube; (2) add 30 mL of KPT80 buffer to the centrifuge tube, cap the tube, and hand-shake vigorously for 2 min; (3) agitate the centrifuge tube ultrasonically for 5 min; (4) centrifuge the tube for 30 min at $1100 \times g$ at 22ºC; (5) label four 250-mL Erlenmeyer flasks 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively, and add 45 mL of sterile LB broth to each, insert a sterile foam cap, and set in a laminar flow hood; (6) transfer 5 mL of supernatant from the centrifuge tube into the 10^{-1} flask and reinsert the foam cap; (7) agitate the contents of the flask using a vortex mixer for 2 min; (8) prepare serial dilutions down to the 10^{-4} range and agitate using the vortex mixer; (9) discard 5 mL of the 10^{-4} flask; (10) incubate the flasks at 37ºC and agitate at 130 rpm for 24 h; (11) after 24 h of incubation, evaluate flasks for visible growth and measure the OD at 560 nm for the greatest dilution with visible turbidity; and (12) refer to the SBU chart to determine the microbial contamination of the slurries.

Microbes are usually quantified in environmental matrices by isolating and quantifying microbial cells, DNA, and ATP. For most environmental matrices, DNA

Table 1. Standard bacterial unit (SBU)* scale.

SBU value	Range of microbial concentration (cell/mL)	Degree of contamination
$\geqslant$3:15 SBU	$\geqslant 6 \times 10^5$	Severe
2:15–3:15 SBU	$3 \times 10^5 - 6 \times 10^5$	Substantial
1:5–2:15 SBU	$1.0 \times 10^5 - 3 \times 10^5$	Low
1:1–1:5 SBU	$1 \times 10^3 - 5 \times 10^4$	Very low
$\leqslant$1:1 SBU	$\leqslant 1 \times 10^3$	None

* explanation in text.

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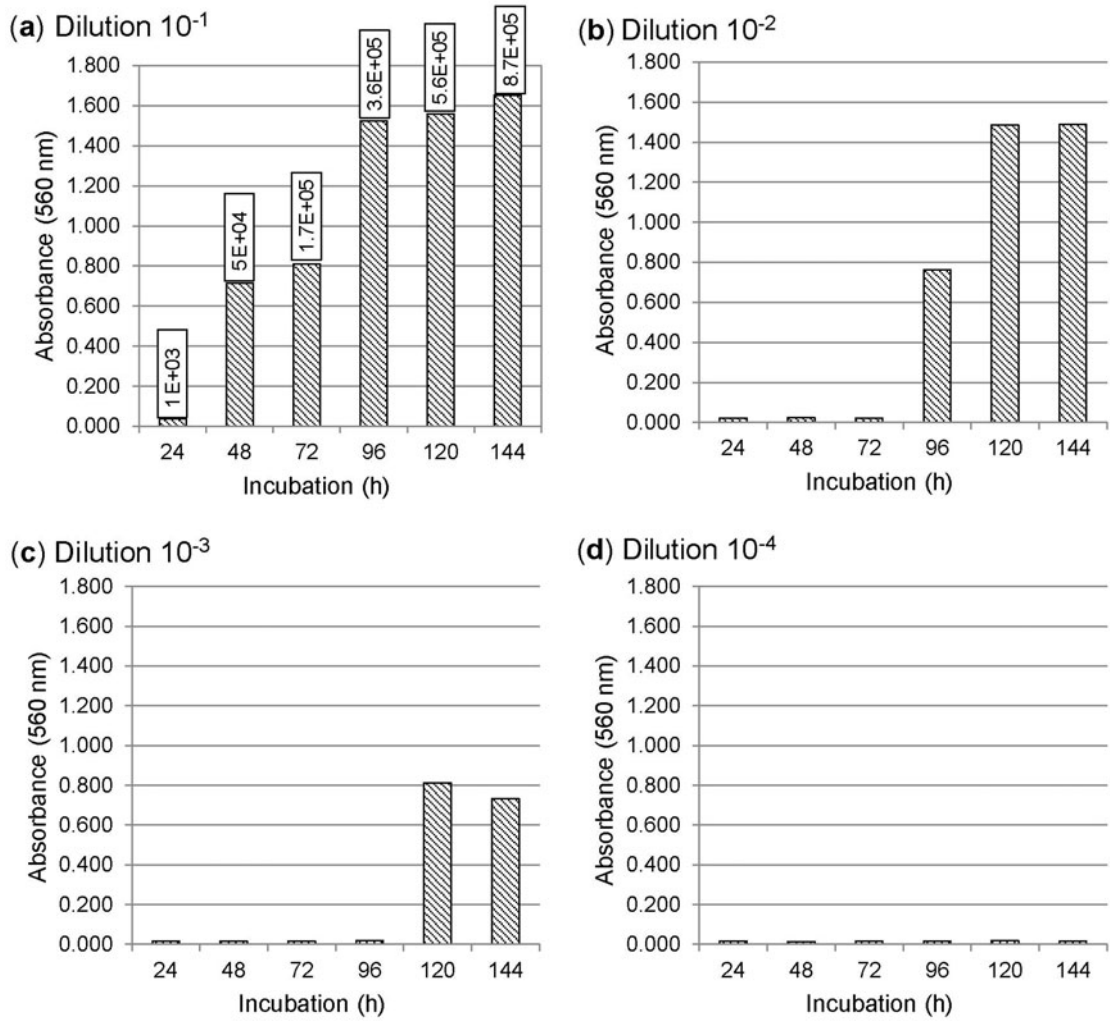


Figure 4. Propagation time and absorbance in (a) 10^{-1}, (b) 10^{-2}, (c) 10^{-3}, and (d) 10^{-4} dilutions. Microbial counts (cells/mL) are presented above the columns in part a.

and ATP methods usually provide better results. Kaolinite chemistry, unfortunately, does not support DNA and ATP methods, *e.g.* clayey soils hinder DNA and ATP extraction (Webster *et al*., 1984; Pangburn *et al*., 1994; Zhou *et al*., 1996). Direct DNA quantification from soils is biased (Frostegård *et al*., 1999) due to the effects of DNA polymer length (Ogram *et al.*, 1994) and soil chemical composition (Zhou *et al*., 1996) on DNA extraction. Clays were reported to exhibit unusually high affinity for DNA (Paget *et al.*, 1992).

More traditional methods of microbial-cell isolation and quantification in environmental matrices are based on direct microbial-cell counts and have claimed to be more reliable tools to evaluate microbial abundance, particularly in highly weathered, clayey, and acidic matrices (Ehlers *et al*., 2008). Direct microbial counts are, nevertheless, limited for kaolinite environments because most microorganisms are attached to kaolinite platelets due to van der Waals forces, electrostatic attraction (van Loosdrecht *et al*., 1987), or strengthened by lipopolysaccharide interactions (Allison and Sutherland, 1986; Chenu, 1993). This study presents a method that combines microbial elution with microbial growth dynamics to successfully measure microbial contamination in kaolinite slurries.

Method validation

A set of 50 kaolinite-slurry samples with various contamination levels was prepared by Imerys and delivered to GCSU for blind testing using the SBU method. Correlation between the SBU method and the dip-slide method results of Imerys was 92%. In four samples, a contamination level of ~10^5 cells/mL was detected by the dip-slide method, but no contamination was detected by the SBU method. These four samples were kept at room temperature by Imerys and analyzed 2–3 days later than at GCSU, which led to propagation of microorganisms in the kaolinite slurries. As a result,

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all further experiments were corrected to prevent the propagation of undesirable indigenous microorganism and this problem did not recur in subsequent experiments. In all other cases, the SBU method provided either identical or greater sensitivity to microbial contamination than the dip-slide method. This was most evident in the analysis of the less contaminated samples (Table 2). The dip-slide method did not detect contamination in ten samples that had 'very low' contamination ($1-5 \times 10^3$ cells/mL) by the SBU method. Similarly, the SBU method revealed 'severe' contamination in samples for which the dip-slide method detected either 'substantial' or even 'low' levels of contamination. Furthermore, the dip-slide method often had substantial deviations between triplicates, which did not occur with the SBU method. In addition, 48 h is required for the dip-slide method, whereas, the SBU method is more efficient and only requires 24 h. Based on the above data, the SBU method was more accurate and reproducible than the dip-slide method (Table 2) or 'instant' read methods, such as ATP luminescence. The ATP luminescence method only indicated none or severe levels of contamination without usable intermediate units (Imerys, pers. comm.).

Microbial growth dynamics has long been the workhorse for quantifying microbial contamination (Andrews and Presnell, 1972). In the present study, aerobic and facultative anaerobic microorganism growth dynamics made it possible to distinguish low, intermediate, and high microbial contamination levels in kaolinite slurries. Aerobic and facultative anaerobic microorganisms dominate kaolinite slurries (Osipov and Turova, 1997; Papp *et al*., 2012), but the extent to which these organisms are responsible for spoilage is unknown. Anaerobic microorganisms in kaolinite slurries could also be involved in kaolinite-spoilage, but cannot be evaluated by the SBU or dip-slide methods. The SBU method can only estimate anaerobic microorganism abundance based on thermodynamic correlations. Overall, this method provides more accurate, reproducible, and rapid information than the dip-slide method currently used by the kaolinite industry, and is, therefore, recommended as an alternative method to quantify kaolinite-slurry microbial contamination.

Table 2. Blind comparison of 46* samples tested using the dip-slide method (IMERYS) and the SBU method (GCSU).

Level of contamination	— Quantity of samples — Imerys Lab	GCSU Lab
Severe	3	10
Substantial	8	2
Low	2	0
Very low	4	14
None	29	20

* Four samples were removed from the set due to discrepancies between storage conditions at Imerys and GCSU laboratories.

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