

## The attached microorganisms on the sand grain surfaces at the Slow Sand Filters.

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### ABSTRACT

This study was carried out in order to investigate mainly the populations of different microorganisms attached to the sand grain surfaces in the slow sand filter beds at Ashford Common Water-treatment works in London area, U.K. using the mechanical shaking technique. It was proved that this technique is one of the more suitable and safe means in extracting microorganisms attached to particles such as sand grains and/or clay particles by applying the scanning electron microscope technique. The scanning micrographs illustrate that there is a direct proportional declination for the numerical densities of the microorganisms at different depths and various time intervals of shaking, while illumination intensities showed a conspicuous pattern particularly in case of complete shading. Simultaneously, It was too difficult to have any other organisms particularly protozoa in the same micrographs which might be referred to the exaggerated abundance of the algal layer "Schmutsdecke", the presence of certain predators (rotifers, chironomid and insect larvae) and the presence of a very weak attachment between protozoa and sand grains.

**Key words:** Bacteria, Protozoa, Scanning Electron Microscope.

### INTRODUCTION

Sand plays an important role mainly in the biological water treatment process where it helps in providing a suitable ecosystem for numerous organisms helping in the removal of various types of pollutants. This occurs also in marine sediments (Montagna; 1982), in traditional water treatment in many countries including Egypt (Galal and Rashed; 1994) at the end of the pre-chlorination stage or in the slow sand filtration at other countries comprising most Europe and USA (Lodge, 1979; Ives and Rajapakse, 1988; Binnie, *et al.*; 2002). Slow sand filtration depends on the presence of a gelatinous dark green algal layer known as the hypogean layer or *Schmutsdecke* in the uppermost superficial layer of the sand which is formed within the first days (10–20 days) of operation and consists of bacteria, fungi, protozoa, rotifera, various aquatic insects and chironomid larvae. The water produced from a well-managed slow sand filters can be of exceptionally good quality with 90-99% bacterial reduction (Galal and Rashed;1994) and more or less some other pollutants (Galal *et al.*,2018 and Galal;2018). Extraction of bacteria attached to the sand particles is faced by a major problem which is the uncertainty of removing all these bacteria by different techniques. Certain methods as mechanical shaking, ultra-sonication and homogenization were applied where the sand sediment was manipulated. The former technique was adopted in the present investigation depending on data obtained by both Goddard (1980) and Galal (1994). This study was carried out to detach bacterial population from sand grains and to follow up the attached bacteria to those grains after various shaking intervals, beside the observation of protozoa as well.

### MATERIALS AND METHODS

Sand samples were picked up according to Goddard (1980) from filter beds at Ashford Common water treatment work, London, U.K. These samples were divided into 1cm<sup>3</sup> subsamples; using a particular coring system, Perspex splitter and cheese wire; which

were placed into individual hemocytometer wells and kept in a fridge at 7 °C awaiting for extraction of microorganisms and the scanning electron microscopic examination. These processes were carried out within few hours in order to minimize errors due to death, multiplication and predation.

Depending on data obtained by Galal (1989), mechanical shaking of the sand grains was more or less a good extracting technique. Sand subsamples were collected from the top 1cm at different beds (uncovered, partially covered and completely covered), mechanically agitated with 10 ml of sterile water via Stuart flask shaker at various time intervals. The supernatant was decanted to count the detached microorganisms. These 10 ml were replaced by another 10 ml and so on in order to have cumulative aquatic detached bacterial and protozoan numbers at various intervals. Few sand grains were collected at the end of each shaking time to be prepared for scanning electron microscopy through the following processes:

**A ) FIXATION:**

Sand grains belonging to the previous filter beds were fixed for three hours in 3% glutaraldehyde in 0.1M sodium phosphate buffer (PH=7.2) within one hour of the sampling. Then, the fixed sand grains were washed by 0.1M sodium phosphate buffer (PH=7.2) twice, each for five minutes.

**B) DEHYDRATION:**

The buffer-washed sand were dehydrated by passing it through a series of ethyl alcohol: 30,50,70 and 95%, each for five minutes. Then, more three washes of absolute ethanol, each for ten minutes, were made in order to ensure that no traces of water were left in the samples.

**C) CRITICAL-POINT-DRYING (CPD):**

Carbon dioxide has been commonly used to achieve this step from the safety point of view ( Grame and Kevin, personal communication, E.M unit at RHBNC). Therefore sand grains were critical-point-dried using Polaron E 3000 CPD apparatus where liquid and gaseous carbon dioxide have the same specific gravity when the temperature and the pressure exceed a certain critical values (32°C and 82 bars respectively). In this situation, the phase boundary inside the vessel of the CPD instrument was disappeared. This allowed the removal of any liquid from organisms without their passing through a phase boundary. This avoids the probability of losing no organisms from the sand grain surface (Goddard, 1980). It is essential to avoid any drying out of the sand grains at any stage, otherwise the organisms may be damaged and the critical-point-drying procedure becomes pointless. Also, rapid decrease of the pressure inside the vessel of the CPD apparatus is forbidden otherwise the organisms will be destroyed.

**D) SPUTTER COATING:**

The CP dried sand grains were placed on stubs coated with adhesive and sputter-coated with Gold-Palidium to provide the sand grains and its microorganisms with an even and thin conductive metallic surface to prevent the electrons from accumulating on the specimens by earthing it and therefore, images can be watched without noisy. This coating process was carried out in the presence of Argon gas and using the Polaron SEM coating unit E 50100. Then, the samples were examined and photographed using the scanning electron microscope (Cambridge Stereo-scan 100). It was convenient that, following the dehydration with absolute ethanol and the CPD procedure, the sand grains could be kept in a dry place until the CPD instrument and the scanning electron microscope were available .

## The attached microorganisms on the sand grain surfaces at the Slow Sand Filters.

### Examination and enumeration of microorganisms attached to the sand grains:

Three different groups of the sand grains were examined carefully using the scanning electron microscope in order to count and size the various forms of the attached microorganisms as follows:

The first assembly of grains was examined to assess the effect of shaking times on densities of the organisms still attached to the sand grains at the same depth. The shaking times tested were 5,10,15,20 and 30 minutes at a maximal speed (40 HZ).

The second set of grains was examined to assess the presence of these organisms on sand grains at the same shaking time but at different depths (1,3,5,10,20 and 30 cm).

The third group of grains dealt with the effect of three different light conditions at 1cm depth as following:

- a) Microorganisms belonging to the uncovered bed{Bed 14}.
- b) Those belonging to the completely covered bed{Bed 5}.
- c) Those belonging to the partially covered {Bed 4}.

Electron micrographs were taken using the camera electronically connected to the microscope. The used magnification ranged from 500 X up to 15 KX.

Statistical analysis was carried out using Minitab<sub>15</sub> statistical program.

## RESULTS AND DISCUSSION

### Organisms on sand grains from different depths:

Plate (1) shows an electron-micrograph of a sand grain magnified 32 times. The grain was collected from bed 14 at 1cm sand depth of a sampling core. The electron-micrograph shows clearly that the attached microorganisms (bacteria) inhabit mainly the crevices and pits of the sand grain surface where they are not exposed to the direct flow of interstitial water. The surfaces of the sand grains exposed to the water flow are covered with a bacterial matrix secreted by the bacterial cells providing a strong attachment. In addition, Plate (1) shows a series of electron micrographs with magnifications of x 2000 which photograph the surface of sand grains taken from various depths of 1, 3, 5, 10, 20 and 30 cm in bed 14. In all six electron micrographs, the area of sand grain surface photographed was  $630 \text{ um}^2$  ( $30 \text{ um} \times 21 \text{ um}$ ). The three common bacterial forms of rods, cocci and fusiform can be clearly observed and are countable, as numbers per  $630 \text{ um}^2$ , while protozoan organisms were not easily detected at all on the sand grain. The results of these counts are given in Table (1) which shows that over 60% of the bacterial cells were cocci and less than 40% were rods plus fusiform bacteria on the sand grains at 1, 3 and 5 cm depths. At 10 cm depth only a third of the total bacteria were cocci and, while at 20 and 30 cm depths, over 90% were cocci. It was obvious that the densities of these bacterial cells decline with depth, resulting in total densities in the deeper sand that is only half or a third of that of the uppermost sand layer. At the maximal density of 213 per  $630 \text{ um}^2$  area, each  $1 \text{ um}^2$  area of sand grain is theoretically occupied by 0.34 of a bacterium.

### Organisms on the grains after shaking for different time intervals:

This test determined the number of organisms left on the sand grain surfaces after 5, 10, 15, 20 and 30 minutes of shaking for sand subsamples at the same depth. Plate (2) illustrates the number and kinds of the attached bacterial cells at the end of each shaking time interval. The data proved that there is a direct proportional decrease with the shaking time in the numerical densities belonging to the different types of attached bacteria with the presence of some filamentous structures, which were not counted. Simultaneously, no

protozoan organisms were detected on the sand grains at the end of the previously mentioned periods which is parallel to data obtained by Galal (2014).

Table (2) shows the remaining number of the attached bacteria at intervals between 1 and 30 minutes, and confirmed results obtained by Galal and Rashed (1994). Also, the same table showed that the remaining number of cocci is higher than that of the rods plus fusiforms at more or less the end of shaking intervals; 95% of the rods and fusiforms had come off, whilst only 85% of the cocci were removed from the sand grains.

### **Effect of bed shading:**

Shading effect was carried out using sand in bed 4 as partially covered one, in bed 5 as completely covered, while bed 14 was studied as uncovered one. The electron micrographs in plate (3) show the bacteria attached to the sand grains taken simultaneously at the top 1 cm sand of these three filter beds. It appears that bacterial matrix is more dense in the micrograph of the uncovered bed 14 than that of the partially-shaded bed 4, while that of the completely covered one (bed 5) shows almost no matrix. A further difference between these beds proved that both the unshaded bed 14 and the partially-shaded one (bed 4) have mostly cocci, some rods and less fusiform bacteria attached to the sand grains, whilst bed 5 has a huge density of fusiform cells together with some prosthicate bacteria (personal communication with Botany Department at RHBNC, London University, UK).

The abundance of fusiform and prosthicate bacteria in the shaded bed 5 implies a greater tendency towards micro-anaerobic pockets than in the other two beds. The reduced 'slime' matrix in bed 5 may be associated with un-favorable conditions for aerobic bacteria which are the main stimulator to secrete this material of "acidic polysaccharide".

Like the preceding two tests, non of the protozoan organisms was observed or detected at any of the electro-micrographs which could be referred to a very weak attachment between sand grain surfaces and protozoa particularly the sessile forms such as *Vorticella*, *Carchesium*, *Opercularia*, *Suctorina* ...etc. which combined with the presence of the dense green algal layer above sand surface of the operational beds. Accordingly, further modification has to be taken in our consideration related to the future work concerning protozoa in sand sediments using SEM. **The numerical densities of detached bacteria and the interstitial protozoa from the sand subsamples proved that both bacteria and protozoa increased within the whole 30 minutes of shaking.** The application of the regression analysis for removed bacteria and protozoa exhibited that the obtained data for both organisms behave more or less similar to each other. It was necessary to keep in mind that the detachment of both bacteria and protozoa from the sand grains was proved to be highly significant ( $p < 0.001$ ) as shown in Table (3).

Simultaneously, the scanning electron micrographs were useful in confirming that the coverage of sand grain surfaces by bacterial cells was greatest in the uppermost sand layers and declined with sand depths. This was supported with data obtained previously (Jones, 1979) using the epifluorescence technique. There was also a visual confirmation on the efficiency of shaking to remove bacteria from the sand grains by mechanical shaking. It was interesting that the highest bacterial density calculated from the micrographs of small portions of the sand grain surfaces was 0.36 cells per  $\text{cm}^2$  at 1 cm depth which declined to 0.12 cells/ $\text{um}^2$  at 30 cm depth. However, the micrographs show that such densities are not uniform over the sand grain surfaces because more bacteria occupy the crevices of the grain as compared with those on exposed surfaces. It was obvious that bacteria attach themselves to the substratum by mucous secretions and thread-like muco-polysaccharides. A further point of interest revealed by electron microscopy is the presence of obligate anaerobic forms

**The attached microorganisms on the sand grain surfaces at the Slow Sand Filters.**

of bacteria in the sand filter that functions entirely aerobically (personal communication with Botany department at RHBNC, London University).

**Table (1) Bacterial densities/ per fixed area of sand grains at different depths using SEM graphs.**

Depth (cm)	Rods & Fusiform bacteria	Cocci bacteria	Total/area *
1	76	137	213
3	56	75	131
5	39	64	103
10	36	16	52
20	4	103	107
30	4	66	70

**Table (2) Bacterial densities per fixed area of sand grains at different time intervals using SEM.**

Shaking time (min.)	Rods & Fusiform bacteria	Cocci bacteria	Total/area *
5	87	40	127
10	12	76	88
15	14	52	66
20	22	10	32
30	8	16	24

\*The fixed area of each micrograph equals  $630 \text{ um}^2$

**Table (3) The detached bacteria and the interstitial protozoa extracted from the sand grains during the mechanical shaking technique.**

<u>The regression equation of detached bacteria using EM</u>						<u>The regression equation of interstitial protozoa</u>					
Time = - 4.45 + 0.817 Bacteria						Time = 33.2 - 23.5 protozoa					
S = 3.14966 R-Sq = 89.7% R-Sq(adj) = 88.4%						S = 3.10045 R-Sq = 90.0% R-Sq(adj) = 88.8%					
Analysis of Variance						Analysis of Variance					
Source	DF	SS	MS	F	P	Source	DF	SS	MS	F	P
Regression	1	692.24	692.24	69.78	0.000	Regression	1	694.70	694.70	72.27	0.000
Residual Error	8	79.36	9.92			Residual Error	8	76.90	9.61		
Total	9	771.60				Total	9	771.60			

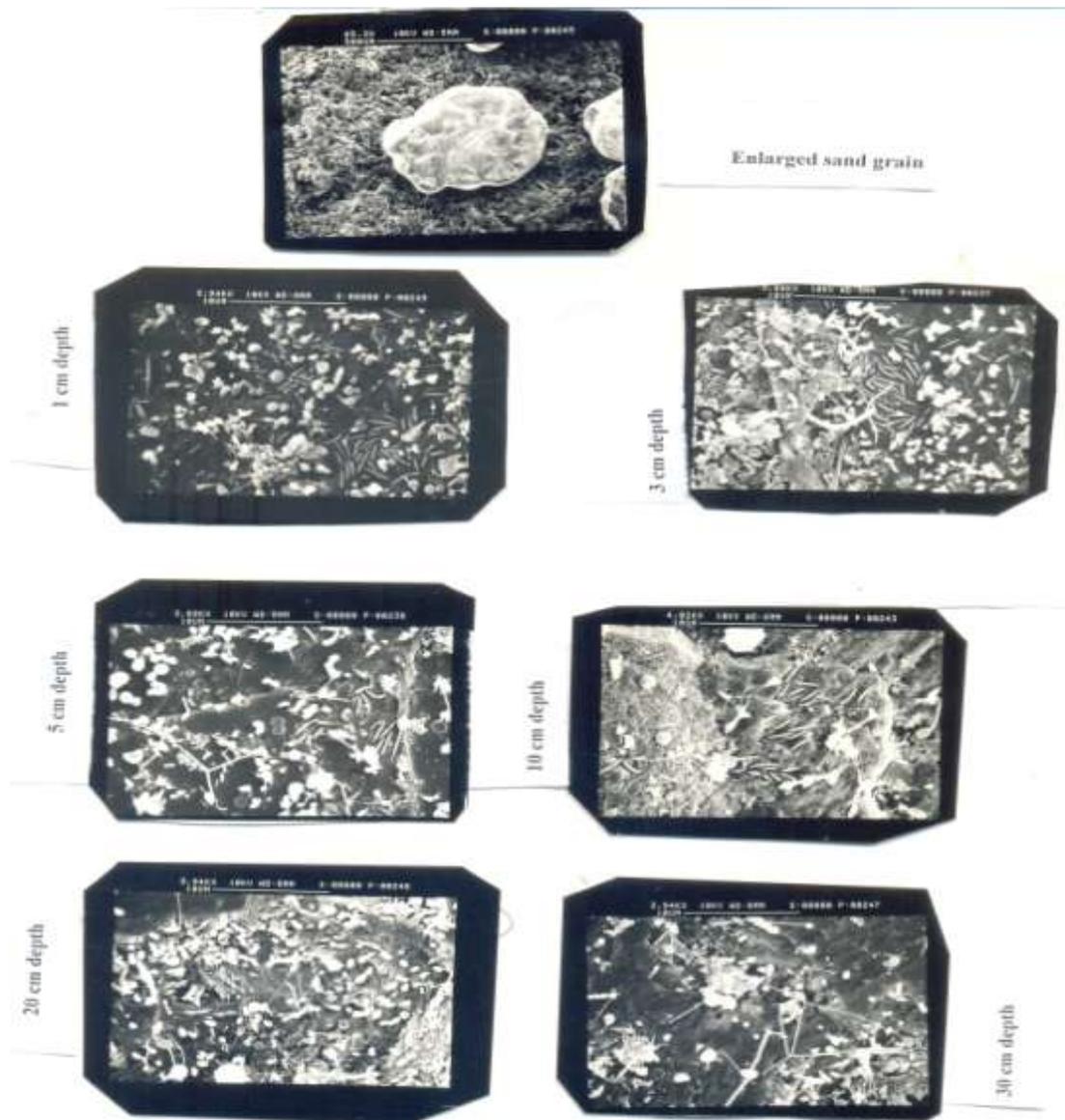


Plate (1) Enlarged sand grain and its attached bacteria at 1, 3, 5, 10, 20 and 30 cm depths.

The attached microorganisms on the sand grain surfaces at the Slow Sand Filters.

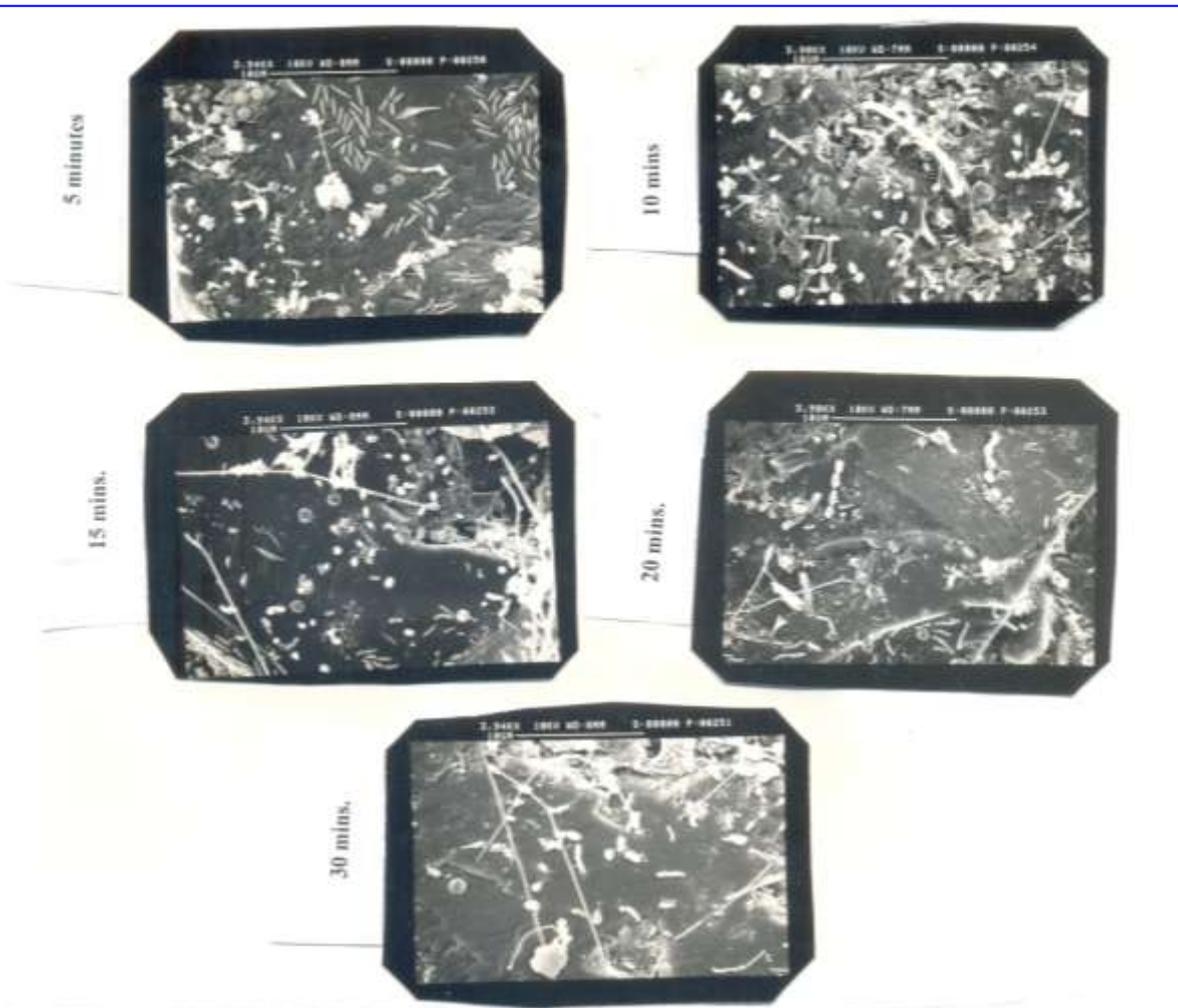


Plate (2) Bacteria attached to sand grain surfaces after 5, 10, 15, 20 and 30 minutes of mechanical shaking.



Plate (3) Bacteria attached to sand grain surfaces at various illuminations.

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## تجمعات الكائنات الدقيقة الملتصقة على سطح حبات الرمل في مرشحات الرمل البطيئة

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أجريت هذه الدراسة أساساً لمتابعة تجمعات الكائنات الدقيقة الملتصقة بحبيبات الرمل في مرشحات الرمل البطيئة بمدينة أشفورد بمقاطعة لندن بالمملكة المتحدة باستخدام تقنية الهز الميكانيكي وفحصها بالميكروسكوب الإلكتروني الماسح. ولقد أوضحت الدراسة تناقص طردى في اعداد البكتريا المتبقية على سطح حبيبات الرمل سواء على اعماق مختلفة أو بعد أزمنة متباينة من تلك التقنية ، بينما كان للإضاءة تأثير متباين وواضح. هذا ولم تتمكن من رؤية أي من الكائنات الأولية على سطح الرمل في نفس الصور السابقة ، ويمكن ارجاع ذلك إلى الكثافة الشديدة لطبقة الطحالب الخيطية الخضراء على الطبقة السطحية للرمل ، كما يمكن ارجاع ذلك إلى وجود بعض المفترسات مثل العجليات ويرقات الحشرات بالإضافة إلى ضعف قوة الارتباط بين الأوليات وحبيبات الرمل وهو عكس ما يوجد بين البكتريا وحبيبات الرمل.