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COMPARISON OF MECHANICAL SHAKING, ULTRASONICATION AND HOMOGENIZATION AS TECHNIQUES USED IN REMOVING ATTACHED BACTERIA AND THEIR EFFECTS ON THE ASSOCIATED PROTOZOA

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مقارنه بين الهز الميكانيكي والموجات فوق الصوتية والطحن كتقينات تستخدم لاستخلاص البكتريا المثبتة وتأثيرها على الكائنات الأولية الموجودة معها

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أثناء دراسة بعض الحيوانات الأولية في المرشحات الرملية المستخدمة في تنقية مياه الشرب بإحدى محطات مدينة لندن بانجلترا وجد أنه من الضروري بمكان دراسة الاهلات البكتيرية البينيه والمثبتة على حبيبات الرمل وذلك لدورها الحيوي مع الحيوانات الأولية في إزالة الملوثات العضوية من مياه نهر التيمز ، ولقد استخدمت في هذا البحث تقنيات طبيعية ثلاث دون اللجوء إلى أية مواد كيميائية وذلك لاستخلاص البكتيريا المثبتة على حبيبات الرمل .

ولقد وجد أن الموجات فوق الصوتية هي الأكثر كفاءة في هذا المجال وذلك خلال الدقائق الست الأولى والتي يظهر بعدها التأثير الضار الذي يؤدي في النهاية لاضمحلال الأهلات البكتيرية ، بينما وجد أن الهز الميكانيكي هو الأكثر أمانا وسلامة بالمقارنة بالطحن والموجات فوق الصوتية لكل من حبيبات الرمل والبكتيريا .

ولقد أثبتت هذه التقنيات أنها متلفة بدرجات متفاوته لخلايا الكائنات الأولية ولهذا توصي هذه الدراسة بعدم إستعمال عينات الرمل المستخدمة مسبقا لاستخلاص البكتيريا لفحص ودراسة الأهلات الأولية وكثافتها العددية .

Key Words: Bacteria, Homogenization, Mechanical shaking, Protozoa, Ultrasonication.

ABSTRACT

During studying of Protozoa in the slow sand filter beds in Ashford Common Water Treatment Work (London, U.K.), it was found necessary to study the interstitial and attached bacterial populations involved with sand particles, due to their vital participating role together with the protozoan populations, in decomposing the organic pollutants from Thames River. Three methods were applied in the present study as they were the most commonly used physical techniques in extracting bacteria. It was found that ultrasonication was very effective in this respect within the first 6 minutes only after which its destructive effect

became highly pronounced leading to a conspicuous declination of the bacterial densities. On the other hand, mechanical shaking appears to be a safer and more convenient technique taking in consideration the destructive effect of homogenization and ultrasonication on the sand grains and bacterial populations. At the same time, it was proved that these techniques were destructive to the protozoan cells by varying degrees and therefore, it was recommended not to use the same sand samples used previously for bacterial extraction to study the protozoan populations.

INTRODUCTION

Enumeration of the bacterial populations attached to the sand particles is faced by a major problem which is the uncertainity of removing all these bacteria by different techniques. Various methods were used in which sediments were manipulated by physical treatments as hand shaking, shaking with glass beeds, ultrasonication and homogenization. The latter two techniques were most frequently used on the assumption that they gave the maximal yield [1, 7, 8, 9 & 10]. Mechanical shaking was used as described by [3 & 4) without using any chemical or surfactant.

A study was made in order to examine the possibility of the protozoan cells to be simultaneously extracted under the application of the above mentioned techniques.

MATERIALS AND METHODS

Sand was collected, from Ashford Common Water Treatment Works near London, using 15 PVC cores refering to [3 & 4) where $10cm^3$ sand subsamples were obtained at 10cm depth. This work was carried out during a period extending between May 1989 and August 1990 in the laboratories of RHBNC - London University and its data were treated and analysed in the Faculty of Science, El-Menofeyia Univ., Egypt using its computer facilities. The subsamples were kept in a haemocytometer tray at 6°C to be analysed within 24 and 48 hours of sampling for minimizing errors caused by multiplication and death of both bacteria and protozoans. Ten cores were choosen randomly from the filter bed and divided into two equal groups; the first one was used for the bacteria and the other was used to study their Protozoa.

For bacteria, fifteen subsamples belonging to five cores were divided into three groups for analysis of the recovery of attached bacteria by ultrasonication, homogenization and mechanical shaking. Each sub-sample was put in a bottle of 50ml capacity and rinsed ten times using 35 ml sterilized filtered bed-water each time to remove the interstitial bacteria. After that, 100 ml of sterile water were added. Ultrasonication was applied using a Decon FS ultrasonication bath; homogenization was performed using ER 10 blender and mechanical shaking was carried out by using Stuart flasks' shaker. Aliquots of 1 ml bacterial suspension were removed from the replicates for counting and was replaced by 1 ml of sterile water to make up the original volumes. The last procedure was repeated at certain intervals (Table 1). Bacterial counts were made according to the epifluorescence technique by using acridin orange [5].

At the end of 30 minutes of both homogenization and shaking and 6 minutes of ultrasonication, a few sand grains were picked up and stained with acridin orange in order to see the residual attached bacteria in each technique. Finally, mechanically shaked and homogenized bacterial suspensions were decanted and the remaining sand grains were covered by 100ml of sterile water and then these latter samples were subjected to ultrasonication for 6 minutes in order to detect its possible additional effect.

For Protozoa, five replicates, each of $10cm^3$ sand were placed in conical flasks of 150m capacity and washed ten times each with 100 ml sterile filtered bed-water and passed through double muslin tissue. Live protozoans were cold sedimented according to [4] in order to be detected and counted. After that, three replicates only were treated by the above mentioned three techniques and their live Protozoa were counted, while the other two replicates were used as control i.e. without treatment.

Due to the long time elapsing in counting alive protozoans, it was impossible to make their count at the same bacterial intervals. Therefore, protozoans were detected and counted after 5, 10 and 30 minutes of homogenization and shaking and at the end of 2, 5 and 10 minutes of ultrasonication.

The data obtained in this study were statistically analysed accordingly to Jones [6] and using Minitab Statistical package.

RESULTS

Table 1 shows that there is a direct relationship between the length of the time of extraction and the detached bacterial densities obtained by mechanical shaking and homogenization throughout thirty minutes. The same pattern holds true up to six minutes of ultrasonication after which a clear declination in the bacterial numbers was obtained. The highest bacterial densities (35.857 and 27.049 $10^7/cm^3$ sand) were achieved after 30 minutes of both mechanical shaking and homogenization respectively, while that of ultrasonication (29.974 $10^7/cm^3$) was obtained at the end of 6 minutes. It appears that 30 minutes of homogenization produced about 90% of that obtained by ultrasonication for 6 minutes and more or less 75% of that yield by 30 minutes of the mechanical shaking technique.

Comparing the effectiveness of these three techniques through regression analysis shows significant results in which mechanical shaking had the highest significant one ($p < 0.005$), followed by those of homogenization ($p < 0.01$) and then ultrasonication ($p < 0.05$) throughout 30 minutes of the former two techniques and 10 minutes of the latter one (Table 2). On the other hand, the same table shows that application of the best regression analysis proves that the best significant results were obtained by 30 minutes of mechanical shaking ($p < 0.005$), 10 minutes of homogenization ($p < 0.005$) and 6 minutes of ultrasonication ($p < 0.005$). It was found that 10 minutes of homogenization yielded 82% of bacteria obtained by 30 minutes of mechanical shaking and 74% of those obtained by 6 minutes of ultrasonication and therefore, the latter technique achieved the highest extracted population within this limited time (6 minutes).

Table 1

Cumulative bacterial densities removed from sand grains at different time intervals of mechanical shaking, homogenization and ultrasonication

Time in Minutes	Bacterial Densities (10^7/cm^3 Sand) + 95% CL of Mech. Shaking	Homogenization	Ultrasonication
1	3.253 ± 2.4	2.947 ± 2.1	4.571 ± 2.5
2	4.937 ± 2.8	4.763 ± 2.7	8.837 ± 3.7
3	6.719 ± 3.2	7.214 ± 3.3	12.728 ± 4.4
4	8.403 ± 3.6	9.875 ± 3.9	17.385 ± 5.2
5	9.561 ± 3.8	11.237 ± 4.2	22.153 ± 5.9
6	11.385 ± 4.2	14.790 ± 4.8	29.974 ± 6.8
7	14.000 ± 4.7	18.129 ± 5.8	28.751 ± 6.7
8	17.497 ± 5.2	19.045 ± 5.5	23.257 ± 6.0
9	20.203 ± 5.6	19.397 ± 5.5	22.940 ± 6.0
10	22.907 ± 6.0	22.164 ± 5.9	19.341 ± 5.5
15	27.114 ± 6.5	24.215 ± 6.1	----------
20	30.795 ± 6.9	26.719 ± 6.4	----------
25	33.428 ± 7.2	26.845 ± 6.4	----------
30	35.857 ± 7.4	27.049 ± 6.5	----------

Studying the data beyond the maximal extracted bacterial densities of both homogenization and ultrasonication proves that the gradual increase in the former technique was not statistically significant ($p < 0.10$), while those of the latter showed an obvious and significant decrease ($p < 0.05$) as can be seen in Table 2.

Table 2

The statistical relationship between the time of extraction of different techniques on the removed sand bacteria under various conditions

Condition	Used Technique	a	b	P
Whole time of extraction	Mech. shaking	5.4	1.2	< 0.005
	Homogenization	8.3	0.8	< 0.01
	Ultrasonication	8.1	1.9	< 0.05
The best regression analyses	Mech. shaking	5.4	1.2	< 0.005
	Homogenization	0.9	2.2	< 0.005
	Ultrasonication	1.2	4.9	< 0.005
Beyond the best regression analyses	Mech. shaking	---	---	< 0.002
	Homogenization	---	---	< 0.100
	Ultrasonication	47.8	-2.9	< 0.05
Subsequent 6 minutes of ultrasonication after 30 minutes of:	Mech. shaking	0.4	0.7	< 0.01
	Homogenization	1.6	2.0	< 0.01

* According to Galal, 1989
Equation is $Y = a + bX$ where
X is the time in minutes. Y is the extracted bacteria.
a is the intercept. b is the slope.

The microscopical examination of the sand grains and their attached bacterial populations after 30 minutes of shaking or homogenization and 6 minutes of ultrasonication, showed that those belonging to homogenization still had considerable numbers of attached bacteria, followed by those related to shaking mechanically, while those of ultrasonication showed the lowest microbial populations which became more or less sparse after a further 4 minutes of ultrasonication.

Table 3 shows that subsequent 6 minutes of ultrasonication for mechanically shaked or homogenized sand samples raised the bacterial yield by about 10% and 37% respectively. This raise was found to be statistically significant ($p < 0.01$) in both conditions.

Table 3

Effect of an additional ultrasonication on the mechanically shaked and homogenized sand grains' cumulative bacterial densities.

Time (Minutes)	Bacterial Densities (10^7/cm^3 Sand)	
	Mech. shaked sand	Homogenized sand
1	0.304	0.950
2	0.782	1.820
3	1.517	3.642
4	2.832	7.751
5	3.218	8.982
6	3.596	10.093

On the other hand, cold sedimentation of the suspended Protozoa showed that zoo-protozoans (54.7%) were slightly higher than phyto-protozoans (45.3%). Among the former group hypostomes and hymenostomes, which are mostly bacterial feeders, formed the largest proportion (35.1%) of the total protozoan organisms, followed by peritrichs (10.5%), spirotrichs (5.6%) and then gymnostomes (3% as shown in Table 4.

Table 4

Number of the most common protozoans/ 10 cm^3 sand cumulated at the end of ten washes

Protozoa	R_1	R_2	R_5	Total	%
Flagellates	620	714	821	2155	20.70
Diatoms	803	890	861	2554	24.60
Litonotus	83	71	90	244	2.30
Hemiophrys	29	18	23	70	0.70
Chilodonella	641	587	639	1867	18.00
Cinetochilum	390	433	461	1284	12.40
Cyclidium	149	173	163	485	4.70
Vorticella con	218	193	207	618	5.90
Vorticella cam	154	137	160	451	4.30
Carchesium	5	4	7	16	0.20
Vaginicola	3	1	1	5	0.05
Stylonychia	11	8	14	33	0.32
Oxytricha	17	29	31	77	0.74
Tachysoma	49	59	61	169	1.60
Euplotes	112	101	93	306	2.94
Amoebae	18	21	18	57	0.55
Total number	3302	3439	3650	10391	100.00

R_n is the number of replicate.
Vorticella con. = *Vorticella convellaria*
Vorticella cam. = *Vorticella campanula*

The main two groups of protozoans show an obvious decline in their densities with the time of extraction (Table 5). The same table shows that zoo-protozoans were more liable to the damage caused by those techniques than phyto-protozoans compared with those at zero time. Ultrasonication was found to be the most destructive technique to Protozoa, followed by homogenisation and then mechanical shaking. The susceptibility to damage was found to be higher in peritrichs than in others.

Finally, examination of many sand grains at the end of the above mentioned techniques showed that no sessile protozoans were still attached to those grains.

Table 5

Effect of mechanical shaking, homogenisation and ultrasonication on cold sedimented live Protozoa at various intervals (minutes).

Protozoa	Techniques And Their Times In Minutes									
	Zero Time	Mech. Shaking			Homogenization			Sonication		
		5	10	30	5	10	30	2	5	10
Flagellates	59	34	16	3	0	0	0	0	0	0
Diatoms	103	93	88	47	83	23	13	48	27	9
Litonotus	17	9	5	6	4	1	0	4	1	0
Hemiophrys	10	6	5	5	2	0	0	1	1	0
Chilodone	91	73	49	13	14	2	2	9	0	0
Cinetochil	72	59	23	9	0	0	0	3	0	0
Cyclidium	40	37	11	4	0	0	0	0	0	0
Vort. conv.	82	69	41	2	0	0	0	1	0	0
Vort. camp.	85	63	13	1	0	0	0	0	0	0
Carchesim	1	0	0	0	0	0	0	0	0	0
Vaginicola	1	0	0	0	0	0	0	0	0	0
Stylonich.	4	2	0	1	0	0	0	3	1	0
Oxytricha	12	6	2	2	4	2	2	9	1	0
Tachysoma	43	29	21	16	21	7	3	4	0	0
Euplotes	45	37	27	23	13	5	3	10	0	0
Amoebae	3	0	0	0	0	0	0	0	0	0
Total	668	517	301	132	141	40	23	92	31	9

Chilodone. = *Chilodonella*
Clinetochil. = *Cinetochilum*
Vort. conv. = *Vorticella convellaria*
Vort. camp. = *Vorticella campanula*

DISCUSSION

Mechanical shaking technique has been applied successfully of 90 minutes to extract maximal bacterial densities from sand grains in the slow sand filtration and it was found that 30 minutes of mechanical shaking extracted 96% of the bacterial densities that obtained at the end of those 90 minutes (3). Similarly, the application of this optimal time in the present study gave the highest yield of the detached bacterial populations (35.857 $10^7/cm^3$ sand). On the other hand, homogenization was applied for periods ranging between 5 and 10 minutes so as to obtain a maximal yield (1, 2 & 9). Actually, the present study proves that there was a considerable yield of detached bacteria up to 20 minutes of homogenisation after which the bacterial populations became more or less steady. This state of stability may be due to certain factor(s) such as the establishment of a balance between the detached and the destroyed bacterial populations.

On the contrary, ultrasonication technique was found to be destructive; while there was a significant increase in the detached bacterial densities ($p < 0.005$) during the first 6 minutes, bacteria were being destroyed at the same time. This was attributed to having a decline in the bacterial populations as treatment continued beyond an equilibrium achieved between extraction and destruction after 6 minutes of this technique (Table 1). Accordingly, extraction by ultrasonication should be kept to a minimum (not more than 6 minutes) due to its destructive nature.

Using the correction factor (1.44) estimated by (2), it was possible to convert bacterial counts obtained by ultrasonication in this study into the approximate numbers originally attached to the sand gains, which was found to be 43.16 $10^7/cm^3$ sand. This estimated original bacterial density was found to be more or less equal to that obtained by each of the other 2 techniques plus those of the subsequent 6 minutes of ultrasonication. Accordingly, it is possible now to have a conclusion that ultrasonication is an excellent technique to yield more bacteria within 6 minutes directly or to estimate indirectly the original attached bacteria to any sand sediment. On the other hand, mechanical shaking takes longer time (30 minutes) but it is a safer technique to be used with a less destructive effect and with a more accurate determination of the bacterial populations on the sand grains.

For Protozoa, mechanical shaking, homogenisation and ultrasonication were found to be destructive to different protozoan groups to varying degrees starting by the fragile forms (peritrichs), where the maximal damage was estimated to be more or less 79.2%, 96.6% and 98.6% (of the number at zero time) at the end of the previous techniques respectively. The resistance of gymnostomes, spirotrichs and diatoms may originate from their flexibility, strong structures as cirri and deposited silica in their walls respectively. Accordingly, identification and/or counting of Protozoa should be carried out using samples not treated by any of the above techniques used for bacterial extraction.

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