Durability and Hygiene of Aspen Tubes Used for Providing Environmental Complexity for Laboratory Rats

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Summary

In Europe the provision of environmental complexity for laboratory animals is mandatory unless there is some welfare-related or scientific reason to prevent their inclusion. Any chemical compound present in the added item to the cage represents a potential confounding factor in the study. The best remedy to this problem is to use a material, such as the wooden bedding material which is already present in the cage. The durability of wooden items means they can be used several times, but they are considered difficult to sanitise. Furthermore, items that are made of several parts may be more easily destroyed than those made of a single unit. This study was designed to explore the durability and possible practical problems associated with sanitation and hygiene of a commercially available aspen tube intended for routine use with rats. The wooden items used were rectangular tubes (20 x 11 x 11 cm) made of dried aspen board with the walls being held together with aspen pins. Before the first use, all of the aspen tubes were autoclaved. At each cage change, the tubes were rinsed either under a pressure washer without detergent or rinsed combined with autoclaving. The tubes were observed for durability and sampled for microbes after use and after sanitation. All of the tubes were discarded before the 14th use. Washing as the sole sanitation method decreased total bacterial burden and coliforms during the first three cycles as compared counts prior to wash. With respect to fungi there were no differences between the sanitation groups. In conclusion, when aspen tubes are cleaned with plain water and pressure, they can be effectively cleaned for up to four cycles. When autoclave treatment is added to the wash cycle, it is the macroscopic damage, which determines the usable life of the item. It appears that aspen blocks can be used in rat cages more than once without any danger of elevating the microbiological burden.

Introduction

In Europe the use of environmental enrichment for laboratory animals is mandatory; unless this is not possible for welfare or scientific reasons (*Council of Europe, 2007; European Union, 2007*). For example, a welfare reason could be fighting between incompatible animals or aggression provoked by items added into the cage (*Kaliste et al., 2006*). Quite simply, if the added item does not improve animal welfare, it is useless. Furthermore, if the added item is suspected of interfering with the study or its interpretation, *i.e.* does not have scientific 'safety', then better solutions have to be sought. This safety aspect is commonly understood as the item being non-toxic, but this is a far too restricted approach.

Any chemical compound added to the cage represents a potential confounding factor in a the study and items added to furnish the cage environment are no exception in this respect. For example volatile

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compounds present in bedding can act as inducers of hepatic microsomal enzymes and this can impact on pharmacological effects *e.g.* duration of drug induced sleeping time (*Ferguson, 1966; Vesell, 1967; Wade et al., 1968; Sabine, 1975; Cunliffe-Beamer et al., 1981; Nielsen et al., 1984; Weichbrod et al., 1988*), but also in some aspects of endocytosis (*Buddaraju & Van Dyke, 2003*). Furthermore it may take several weeks for the enzyme activities to return to normal once the animals are on a different type of bedding (*Davey et al., 2003*). A more recent example is bisphenol A leaching from polycarbonate equipment (*Howdeshell et al., 2003*) having estrogenic effects on the animals living in the cage (*Krishan et al., 1993*).

Items made from organic materials have been shown to emit volatile compounds including pinenes, but this problem can be solved by prior heat treatment (*Nevalainen & Vartiainen, 1996*). Nonetheless, the best way to overcome exposure of animals to new chemical compounds is to use a material already present in the cage or to use genuinely inert materials. One solution would be to use items made of bedding material (*Eskola et al., 1999*), or combinations of bedding and diet (*Kemppinen et al., 2008*). If hardwood chips are used as bedding, then the logical approach is to use the same hardwood to build items or structures which could be placed in the cage to enrich the environment. Moreover, rats seem to like wooden, chewable objects in preference to a diverse group of other items (*Chmiel & Noonan, 1996*).

Wooden items are believed to be difficult to sanitise, and hence regarded as being good only for single use and then being disposed. This, however, is a wasteful and expensive practice. The durability of the wooden items may well exceed a single use, provided they do not represent a source of contamination. There are few recommendations for cleaning the items added to cages, perhaps due to the wide variety of materials that have been used (*Coviello-McLaughlin et al., 1997; Smith & Hargaden, 2001*).

Obviously the animal facility must decide whether the items are to be changed at the same time as the cage, and if not, how should they be washed or autoclaved. It is clear that items that are made of several parts may be more easily destroyed during cleaning and sanitation than those made of one. Furthermore, rodents will gnaw wooden items (*Chmiel & Noonan, 1996; Eskola et al., 1999*) and this may also decrease the durability of items made of wood. This study was designed to explore the lifespan, the practical sanitation and associated hygiene problems of the routine use of commercially available aspen tubes for rats.

Materials and Methods

The study protocol was reviewed and approved by the Animal Care and Use Committee of the University of Kuopio. The durability of aspen tubes (Study 1) was studied in two facilities: National Laboratory Animal Center of the University of Kuopio and Laboratory Animal Centre of the University of Oulu. The study protocol was similar in both laboratories. The hygienic study (Study 2) was done solely in Oulu.

Study 1. The durability of aspen tubes

Aspen tubes

The wooden items were rectangular tubes (20 x 11 x 11 cm, 1.5 cm wall thickness, Figure 1) made of

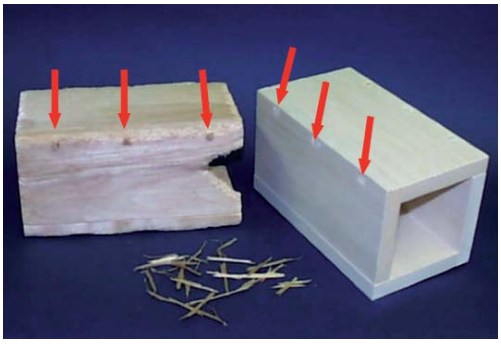

Figure 1. Picture of new (right) and partly gnawed used tube (left). Fragment of aspen wood from other tubes appear at the front of the tubes. The three pins joining the boards at each corner are shown with the red arrows.

dried aspen board (*Populus tremula,* Tapvei Oy, Kaavi, Finland). The walls of the tube were pinned together with aspen pins (4.0 x 0.6 x 0.6 cm) in predrilled holes, *i.e.* no glue was used. Altogether 120 tubes (60 in each facility), identified with a number, were used in the experiment.

Animals and environment

In Kuopio, a total of 448 barrier bred outbred male Wistar rats (WH, Hannover origin) in 112 cages used the tubes. During the study, the rats were 4-11 weeks old, weighing 60-310 g. The rats were housed in solid bottom stainless steel cages (48 x 29 x 20 cm) in groups of four. In Oulu, 440 barrier bred outbred Sprague Dawley rats (Mol:SPRD) participated in the study. The age of the rats varied between 5-14 weeks and the weight ranged from 97 g to 363 g. There were four rats in each solid bottom polycarbonate cage (55 x 35 x 20 cm, Makrolon®). The cages were allocated randomly into racks with other rat cages. The experimental rats were not accustomed to aspen tubes prior to the study in either facility.

In both animal units, the ambient temperature was 21 ± 1 °C and the relative air humidity (RH) 55 ± 10 %. The automatic light and dark cycle of the animal rooms was 12 hours light and 12 hours dark, lights on at 07.00 and off at 19.00 hours. Pelleted rat food (Kuopio: R36, Lactamin Ab, Stockholm, Sweden; Oulu: RM3, SDS, Essex, England) and tap water in polycarbonate bottles were available *ad libitum*.

Aspen bedding (Tapvei Oy, Kaavi, Finland) was used in both units. Since the sizes of the cages differed between the two facilities, the volume of bedding was equalized to 1.2 ml/cm^2 of cage floor area. Cages, bedding and water bottles were changed twice a week.

Study protocol

Before their initial use, all aspen tubes were autoclaved. During the cage change, a tube would be placed into the rat cage, always the same, coded side upwards. In the next cage change, that tube was removed from the cage, washed with a pressure washer without detergent (to avoid any residues) and autoclaved for 12 min at 134 °C with a drying time of 5 min. After this sanitation process, the tubes were again returned to the cages at random. One cycle in use was the time between the routine cage changes, *i.e*. 3 to 4 days.

The tubes were observed at each change for durability in terms of the cracking of the wooden material as well as loosening of the aspen pins and joints. The tubes were removed from the experiment when they were either completely or nearly broken. The number of the cycles passed in use was recorded.

Study 2. The hygiene of aspen tubes after use and transport

Animals and environment

The animal stock was the same as in study 1, but the rats were not the same. The animal care and housing were identical to the Study 1 in Oulu. A total of 40 rats, in groups of four, were involved. At the beginning of the experiment, the rats were 6 weeks old.

Aspen tubes and the experimental protocol

The same type and quality of aspen tubes were used as in Study 1. Altogether ten tubes were divided into two tube-sanitation groups:

1. Rinsing and then leaving to dry in open room air after each use
2. Rinsing and autoclaving after each use

A coded aspen tube was inserted into each cage at the cage change and subsequently used for 3.5 days till the next change. After a new sanitation and sampling, the tubes were returned back to the same cages.

Microbiological sampling and cultivation

At each cage change, the samples were taken first from dirty, used tubes. After the first sample, the tubes in group 1 were thoroughly rinsed with a pressure washer (Kew Alto 4040 CA, Kew Alto,

Billund, Denmark), using municipal water but no detergent. The tubes were left to dry at normal room temperature for 24 h and the microbiological sampling was repeated. The tubes in group 2 were sampled and washed similarly, but they were subjected to autoclaving for 19 min at 121°C with a drying cycle of 11 min. Since the cleaning, autoclaving and drying took over 24 h, the tubes re-entered cages at the next cage change. Meanwhile, there were substitute tubes, not part of this study, placed in the cages.

Microbiological sampling and cultivation followed the recommendations of the Nordic Committee on Food Analysis (*1987*). The samples were always taken from the same part of the aspen tube. The area of 10 x 10 cm was measured at the inside bottom (inside floor) of the tube, always at the same end. A sterile moist cotton-wool swab was rubbed through this whole area, sampling also the sidewalls of the tube to a height of 1 cm. This was repeated three times changing every time the direction of rubbing and then the swab was inserted into the test tube containing diluting liquid.

Three types of microbial analyses were performed. The total bacterial count was chosen to represent the hygienic status of the tubes. Coliform bacterial counts were used as indicators of faecal contamination. Fungi and yeasts have been shown to grow in wooden bedding material, and since these microbes may form toxins, the samples were also processed for these microbes. The samples were cultivated on Plate Count Agar (PCA), for 72 h at 30 °C for total bacterial counts; on Violet Red Bile Agar (VRB) for 24 h at 37 °C for coliforms; and on Saboraud Agar for 168 h at 20 °C, for fungi and yeasts. The number of total bacteria, coliforms, fungi and yeasts are given as colony forming units (CFU).

Microbes before and after transportation

A pilot trial was undertaken to determine whether there would be microbial contamination of the tubes after manufacture but before despatch, after transport or finally after storage in the animal facility.

After manufacture of the tubes, they were heat treated for 10-15 min at 100 °C, then stored for up to two days in cardboard boxes. The temperature in the factory storage room was 15-20 °C and the RH below 60 %. Before transportation, the boxes were wrapped with plastic. The transport took place together with bedding bags, in a covered, solid wall trunk. The transportation distance in this trial was 400 km and the total transportation and storage time was about 24 h.

A total of ten tubes were sampled for microbes and surface moisture. The surface moisture was measured to determine if there was any correlation between the moisture and the microbiological growth. The surface moisture was measured with a moisture meter Humitest MC-100 S (Humitect OY, Helsinki, Finland). Each tube was measured twice at a total of five different points on all sides of the tubes and inside the tube before the microbiological sampling. The measuring was done by pressing the head of the moisture meter onto the surface of the tube. The results were expressed as % RH.

Microbiological sampling was done with the same method as described previously. The first sample was taken in the factory before dispatch. After transportation, the tubes were unpacked in the animal facility and sampled again. In the facility, the tubes were stored in the bedding storage room (22 ± 1 °C; RH 45 %). Additional samples were taken after one and two weeks in the storage room.

Data analyses

In Study 2, CFU/100 cm^2 values were calculated from each sample for total microbes, coliforms, fungi and yeasts. The effect of the sanitation procedure was tested in both sanitation groups after each cycle in use by comparing the microbial counts after use to the counts after tube sanitation. Comparisons were made using Wilcoxon Signed Ranks test. The differences between the two cleansing methods were tested with Mann-Whitney U-test, by comparing the microbial counts of tubes in the washing group to those of washing and autoclaving, after each use and sanitation cycle.

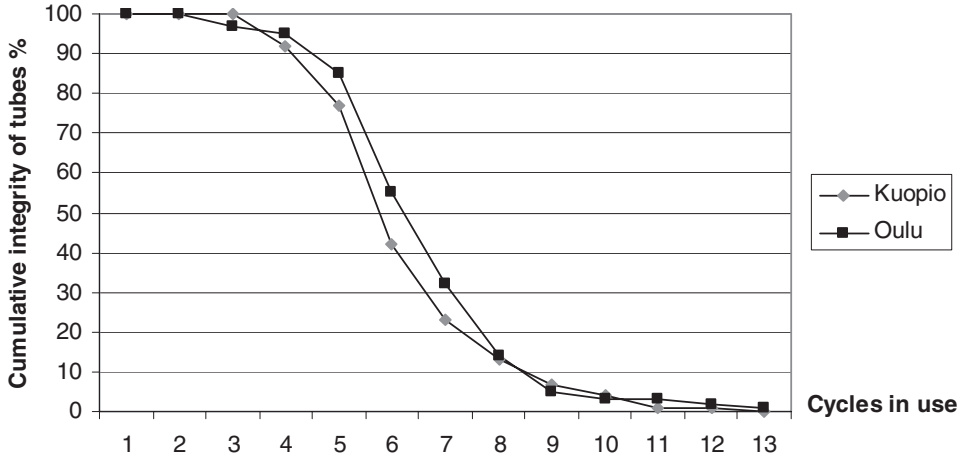

Figure 2. Cumulative mean disintegration of aspen tubes (n = 120) after each cycle (= 0.5 weeks). The tubes were autoclaved before use and after each cycle in use.

Relative humidity (RH) results were calculated with Linear mixed model using tube as the subject and time point as the main effect.

Results

The durability of aspen tubes

All the tubes were broken before the 14th use cycle. The half life of the tubes was about six cycles in both facilities (Figure 2). The rats gnawed the tubes, but none of the tubes broke because of excessive gnawing. In contrast, all of the tubes became unusable because the pins came loose.

Microbe counts and the effect of sanitation

The total bacterial counts after use and after sanitation are shown in Figure 3 and the corresponding coliform values in Figure 4. The growth of different fungi was insignificant and there were only a few, single CFU of these microbes.

The effect of sanitation was assessed in both groups. Rinsing as the sole sanitation method decreased significantly ($p < 0.05$) total bacterial burden at 1st-4th and 6th cycle as compared with the respective prior-to-wash values, but there was no effect on the 5th, 7th or 8th cycle (Figure 3). Simple rinsing decreased coliform values between the 2nd-4th cycles ($p < 0.05$, Figure 4). When the rinsing was complemented with autoclaving no microbial growth was seen. Since fungi were not really a problem there were no differences between the effectiveness of the two types of sanitation.

Comparison of total bacterial counts between the two sanitation groups after each use cycle, but before sanitation, showed a single significant ($p < 0.05$) difference found at the 2nd cycle where the wash-with-autoclave group had higher CFUs (Figure 3). After the sanitation process, plain rinsing was less effective ($p < 0.01$) than rinsing with autoclaving from the 4th cycle till the end of the trial (Figure 3). Again for fungi and coliforms, there were no differences between counts with respect to sanitation procedure (Figure 4).

The hygiene and humidity of aspen tubes from manufacture to storage

There was no growth of bacteria or fungi before or after transportation, or during the first week in storage. Subsequently two tubes out of ten showed one bacterial, and three tubes exhibited one fungal colony on the second week when these were cultured on Petri dishes. There was a significant (p

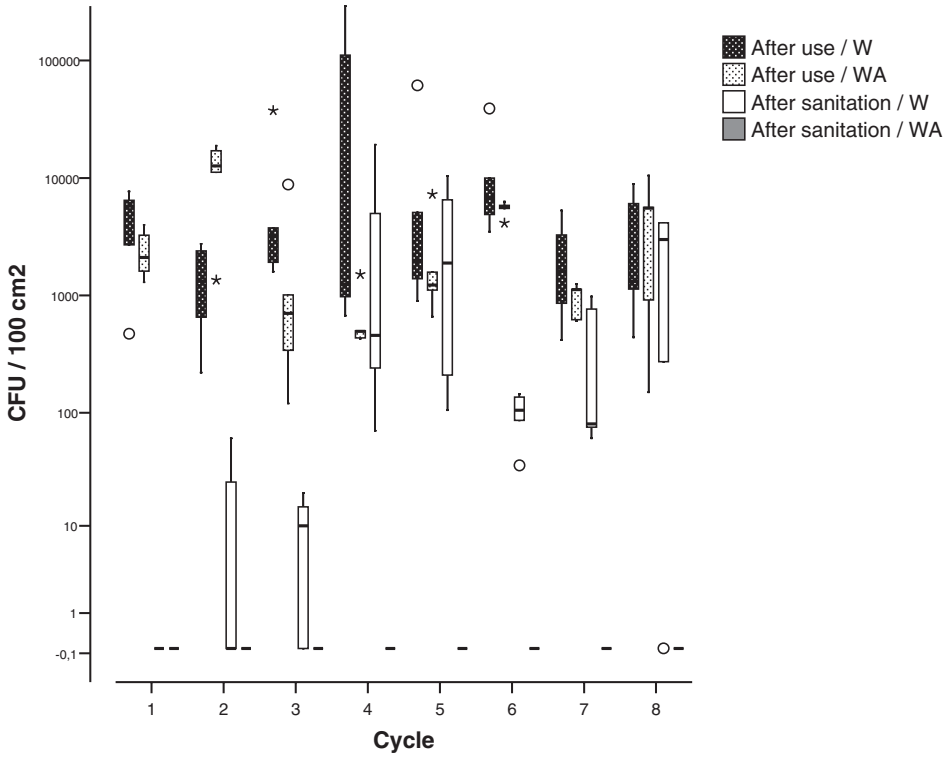

Figure 3. Total bacteria counts (CFU / 100 cm^2) of aspen tubes after use and after sanitation in wash only (W) and in wash and autoclaving (WA) groups. When autoclaving was used, values were always zero in 'After sanitation / WA' group. Each box represents five tubes showing the median, quartiles, outliers and extreme values within a category.

< 0.001) difference between RH values after transportation as compared to the corresponding values after two weeks of storage in the facility; RH increased from 7.6 % to 8.3 %.

Discussion

In principle all new materials to which animals are exposed in the cage may interfere with a study or interpretation of its results. Traditionally pesticide residues and heavy metals have caused concern as opposed to chemicals normally present in the cage. Unless the confounding potential of all chemicals is known, the best remedy is to utilize only materials already present in the cage or inert materials. This aspect is widely ignored when items are placed into cages for enrichment purposes.

Which materials are already in the cage? Older cages are made of polycarbonated plastic, which can hardly be considered inert because these kind of plastics are known to leach breakdown products, which have estrogenic effects (*Howdeshell et al., 2003; Krishan et al., 1993*). Water bottle caps and sometimes the cages themselves are made of stainless steel (*Voipio et al., 2008*), which can be considered to be an inert material. The feeding method may not come into mind at first sight, but food pellets have been used in diet boards made of wood. These boards can been used to control obesity, but also contribute to cage structural complexity (*Kemppinen et al., 2008*). Items could be

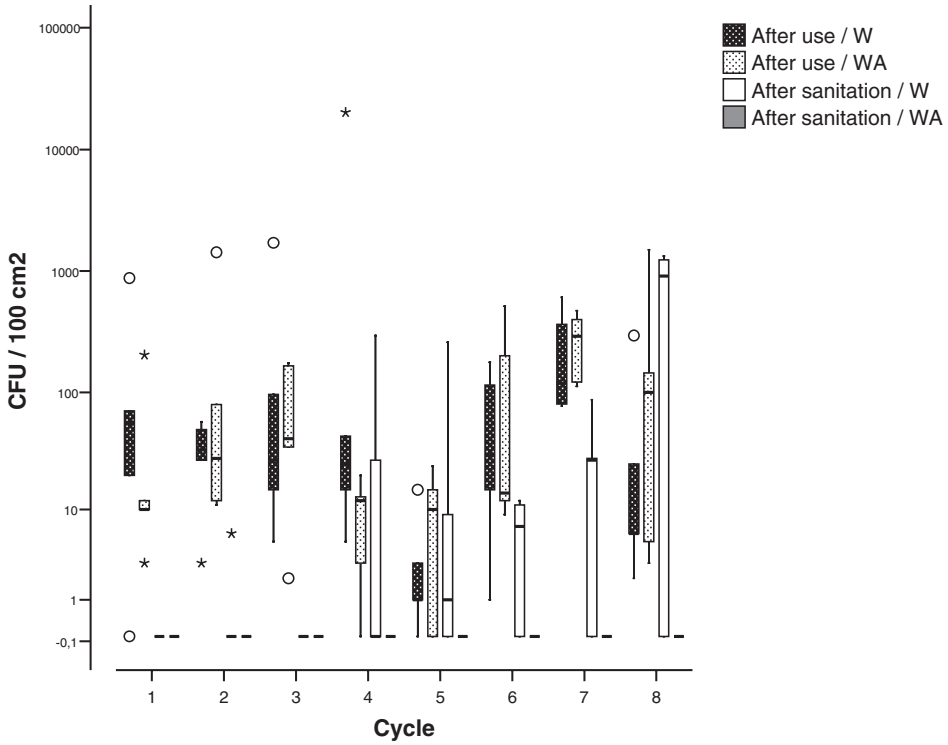

Figure 4. Coliform counts (CFU / 100 cm^2) of aspen tubes after use and after sanitation in wash only (W) and in wash and autoclaving (WA) groups. When autoclaving was used, values were always zero in 'After sanitation / WA' group. Each box represents five tubes showing the median, quartiles, outliers and extreme values within a category.

constructed from the same wooden material as used for bedding chips. This would seem to be the most logical and obvious choice for building furniture elements to be introduced into cage, and this is the reason why an aspen item was chosen in this study. One of the most common bedding materials is wood as sawdust or chips of variable particle sizes. This same material in the form of wooden board can be used to create various structures, such as dividing walls, nest boxes, shelters and tunnels. A hardwood material, like aspen, is recommended to avoid unwanted metabolic effects. If wood items are used only once, and then thrown away, no sanitation is needed, nor is the structural durability of the item a problem. However, this kind of practice is wasteful, and does not provide the olfactory clues preferred by the animals. This study has evaluated the most common sanitation options for aspen tubes and the impact of these procedures on the durability of the items, and in this way trying to clarify which is the decisive factor in the use of these enrichment items. The half life of the autoclaved tubes - six cycles - is evidence that they are suitable for multiple use in routine operation. Autoclaving results in considerable wear and tear on the tubes, as opposed to plain pressure rinse where the limiting durability factor is the amount of wood gnawed by the rats. After autoclaving it seems to be the wooden pins, which keep the tube together; these represent the weakest link. An attempt to solve the latter problem

with thicker pins was made during this study, but without success.

Autoclaving as a process leads to a ten-fold decrease in the amount of volatile compounds - including pinenes - emitted from bedding material (*Nevalainen & Vartiainen, 1996*), and this serves as a convenient option to ensure that these materials do not interfere with an experiment. Whether the situation is similar with larger board pieces is unclear. Items to be added into the cage should be treated similarly to the bedding because items made of organic materials may contain also volatile compounds, such as pinenes.

Autoclaving, even plain water rinsing, are bound to change the olfactory clues carried over to the next cycle of use. Hence, a good sanitation practice does necessitate marking items in order to make sure that they return to the cage of origin. This is important because secretions, particularly urine, contain an enormous amount of olfactory information; this is how rats can identify the animal that produced the odour (*Agosta, 1992*). An increasing urinary smell as the material becomes impregnated with urine may be revolting to humans, but how the rats may perceive this sensory cue remains unknown.

Wood is a porous material, and hence it is difficult to sanitise in order to maintain good hygiene. On the other hand at some point it is clearly essential to sanitize the items. Solutions meant for nonporous materials, such as a tunnel washer (*Smith & Hargaden, 2001*) or cold sterilisation (*Coviello-McLaughlin et al., 1997*) cannot be used, because detergent or the sterilant may adhere to wood. In this study, the items were removed for cleaning at every cage change, *i.e.* after being in use for half a week. In the hygienic evaluation, total microbial counts and coliforms from surface swabs proved to be the most useful. It appears that rinsing alone was good enough to maintain a reasonably good level of hygiene until the fourth cycle, *i.e.* two weeks use can be reached with a plain pressure rinse (Figure 3).

The growth of fungi was only marginal, and it has been shown that aspen chip bedding - even when purposely contaminated with fungi - does not favour the growth of fungi in the rat cage environment (*Pernu et al., 2000*). In hygienic terms aspen boards are better than aspen chips; total bacterial counts in aspen bedding increase exponentially after four days in the rat cage (*Haataja et al., 1989*). The difference may be due to the hugely greater surface area of the chips providing a better environment for microbial growth. Furthermore, bedding chips cannot be cleansed with water.

Hygiene appeared to remain satisfactory for a longer period with a combination of wash and autoclaving. An anecdotal finding during the study was the smell of rat urine, which clung to the tubes after a few cycles, which may be unpleasant to animal care personnel. Nonetheless, autoclaving was destructive to the structural integrity of the item and to the number of times it could be re-used. In practice the point where the item could no longer be re-used can be judged visually.

Relative humidity (RH) stayed low throughout the period from manufacture through two weeks in facility storage. The statistically significant increase in RH was so small as to be unimportant, the critical point is that the RH should not become sufficiently elevated to permit microbial growth. The presence of a few isolated colonies on cultivation appears accidental.

In conclusion, when aspen tubes are cleaned with plain water under pressure, they can be effectively cleaned for up to four cycles. Thereafter maintenance of proper hygiene requires subsequent autoclaving. Considering the macroscopic breakdown of the items in, and labour associated with, autoclaving, it may be enough to use pressurized water for sanitation up to four cycles, and then dispose of the items.

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