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Cork stoppers industry: defining appropriate mould colonization

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Abstract

Aims: The main aims of this work were the study of cork slabs moulds colonization and the evaluation of the moulds diversity during cork processing steps, in different cork stoppers factories. Simultaneously, it was envisaged to perform an evaluation of the air quality.

Methods and Results: Moulds were isolated and identified from cork slabs and cork samples in four cork stoppers factories. The identification was based on morphological characters and microscopic observation of the reproductive structures. Airborne spore dispersion was assessed using a two stage Andersen sampler. It was observed that *Chrysonilia sitophila* was always present on cork slabs during the maturing period, but mould diversity appeared to be associated to the different factory configurations and processing steps.

Conclusions: Spatial separation of the different steps of the process, including physical separation of the maturation step, is essential to guarantee high air quality and appropriate cork slabs colonization, i.e. *C. sitophila* dominance. The sorting and cutting of the edges of cork slabs after boiling and before the maturing step is also recommended.

Significance and Impact of the Study: This study is very important for the cork stopper industry as it gives clear indications on how to keep high quality manufacturing standards and how to avoid occupational health problems.

Key Words: cork stopper industry – mould colonization – wine quality – mould identification – occupational health

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Introduction

Cork stoppers are made from bark from the cork oak tree, *Quercus suber* L. This species is grown in Portugal, Spain and other Mediterranean countries. Portugal is the world largest producer of cork stoppers for the wine industry therefore all the research performed to enhance productivity and quality standards is of the utmost economical relevance.

Lee and Simpson (1993) summarised the manufacturing process of cork stoppers. The process includes a cork slabs stabilisation period, after boiling, during which mould growth completely covers them. This process has been used for many decades, and cork slabs were considered to be of good quality for stopper manufacturing only when they were completely covered with white or salmon-coloured moulds. The role of mould development was not clearly understood.

The micro biota associated with cork during the manufacture process of stoppers is difficult to define. Most of the published studies report that *Penicillium* and *Trichoderma* are the predominant moulds, but this information is difficult to generalize since the methods for species isolation differ, and sample transport conditions are not usually reported. The environmental conditions during the successive steps of the process, particularly those concerning the cork slabs maturing stage, are not usually considered in the literature. Lacey (1973) and Danesh *et al.* (1997) have performed microbiological analysis in Portuguese cork stopper industries to identify the mould species associated with each step of the cork manufacture process. Both authors reported the dominance of *Chrysonilia sitophila* (Mont.) Arx

over the cork slabs during the maturation period after boiling, although Lacey (1973) also described a significant occurrence of *Penicillium* sp. The differences reported by those authors may be attributed to the different industrial practices used during the maturing stage.

The manufacturing processes of the cork stoppers industry has been reported as the main responsible for the appearance of "cork taint" in wines. It was admitted that moulds were the main responsible for "cork-taint" in wine and it was suggested that their development should be suppressed during the slabs maturing stage. The incidence of respiratory problems (asthma and suberose alveolitis) in cork stopper industry workers, attributed to mould spores dispersion into the atmosphere during the slabs cutting after the maturing stage, has further contributed to this assumption. In fact, the pathogenesis of those diseases was attributed to the spora of *Penicillium glabrum* (*P. frequentans*) disseminated in the air of cork stoppers factories (Lacey 1973; Ávila and Lacey 1974).

The goal of this work is to study the main factors responsible for the colonization and diversity of moulds associated with the different production steps of cork stoppers in order to guarantee a high quality for cork stoppers and to improve environmental hygienic conditions.

Materials and methods

Cork stopper factories

Four industrial units of cork stoppers processing were studied. The main steps of cork slabs transformation into cork stoppers followed in the four factories are described in Fig. 1.

The major differences between factories are: (a) factory A makes the sorting and the removing of slabs edges before the maturing stage; (b) factories B_1 + B_2, although geographically separated, are associated in a way that the boiling and the slabs maturing stage are made in B_1 and the other manufacturing stages are made in B_2; (c) factory C includes an additional drying stage before the dimensional correction procedure.

Sampling schedule

The choice of the sampling points took into account the type of facilities studied, the processing line and associated steps, and also the evaluation of the areas with higher levels of airborne particles and spore dispersion. Fourteen sampling points were thus considered: (a) Slabs boiling area; (b) Sorting and removal of cork slabs edges; (c) Maturation area; (d) Strip cutting area; (e) Punching area; (f) Pre-drying area; (g) Dimensional correction area; (h) First electronic sorting area; (i) Washing area; (j) Drying area; (k) Second electronic sorting area; (l) First manual sorting area; (m) Second manual sorting area; (n) Final treatment and packing area. Three or four sampling visits, with at least a month difference, were made to each factory.

Culture media

The culture media used were Wallerstein Laboratory Nutrient Agar – WLN; Yeast Extract Glucose Chloramphenicol Agar – YGC, Malt Extract Agar – MEA, Potato Dextrose Agar – PDA, Oxytetracycline Glucose Yeast Agar – OGY (all from Merck, Darmstadt, Germany) and Dichloran Glycerol Agar Base – DG18 and Czapec Dox Agar modified – CYA (both from Oxoid, Basingstoke, UK).

Airborne spore assessment

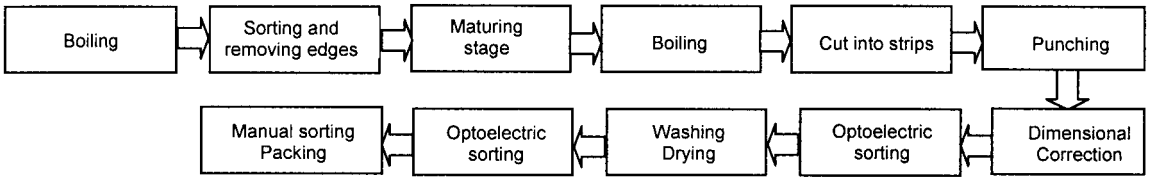
Airborne spore dispersion was assessed using a two stage Andersen sampler (Graseby Andersen, USA) as already used by Lacey (1973). In order to simulate the exposure of individual workers, the air was sampled at the level of the workers shoulder, in each sampling visit. Sampling was performed at a rate of 0.28 m^3 min^{-1}, during 20 seconds, to Petri dishes, containing 20 ml of DG18 agar. The Petri dishes were incubated at 14 °C ± 1 °C in the dark, for 5 days, and were observed daily. After incubation, colony-forming units (CFU) were counted and the total numbers were calculated, as proposed by the manufacturer of the equipment. The results were expressed as the number of viable particles per m^3 of sampled air. For each sampling date, negative controls were performed for the culture media used.

Microbial analysis of cork samples

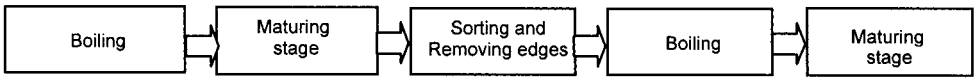
Moulds sampling was performed following two different approaches: a) direct isolation of moulds from cork slabs and b) extraction and quantification of moulds from cork samples.

Direct isolation of moulds. During the maturing stage of the cork slabs, they were completely covered by moulds. Isolation of those moulds was achieved by direct sampling of hyphae or spores from all the different mould growths observed on the slabs, followed by point inoculations on different solid culture media. During the isolation procedure the following solid media were used: DG18, OGY, WLN and DG18 supplemented with of 250 mg l^{-1} Rose Bengal (RB, Sigma, St. Louis, USA). The cultures were incubated at 27 °C and 14 °C in the dark. To check the purity and charac-

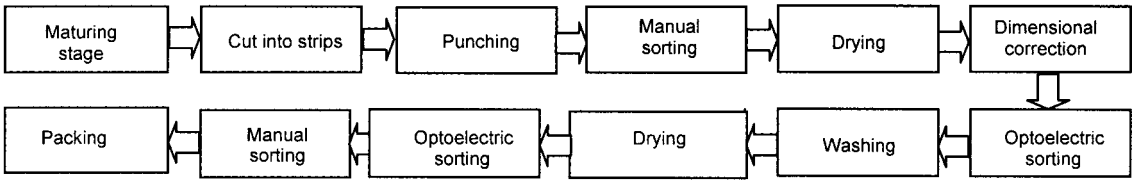
Factory A



Factory B_1



Factory B_2



Factory C

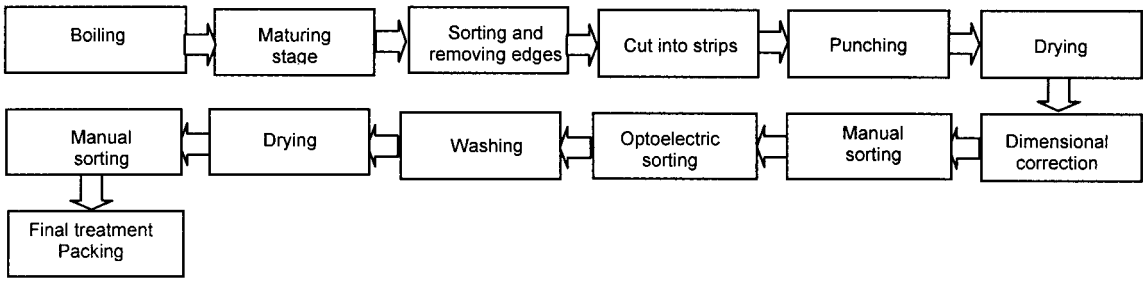
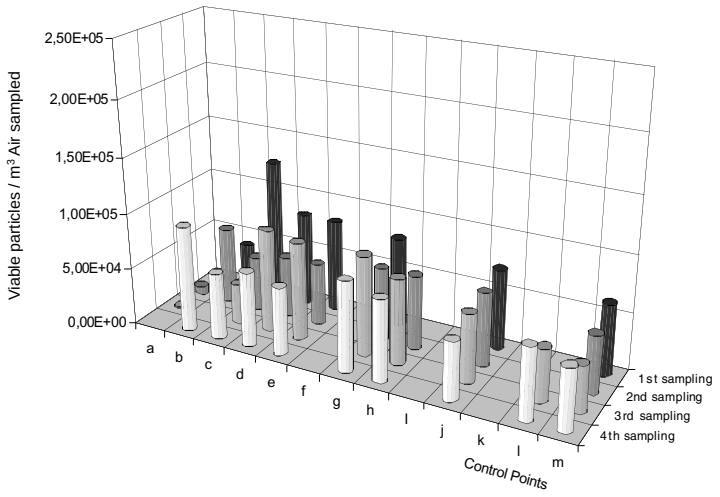


Fig. 1. Processing steps of the different manufacturing plants sampled.

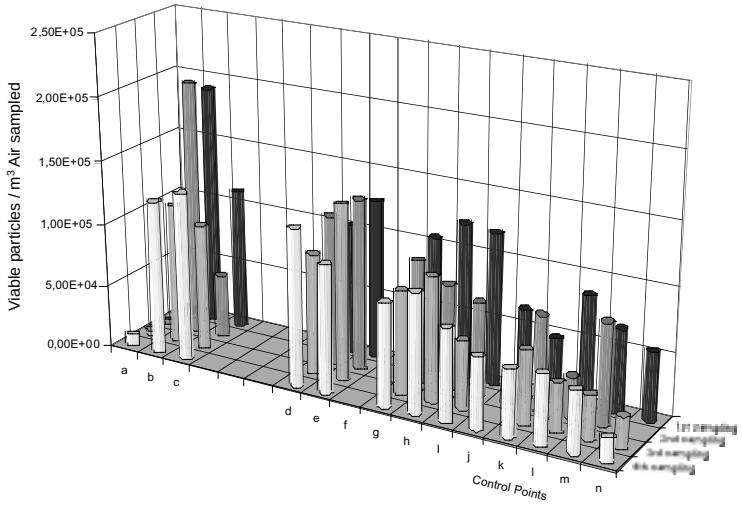
terise the moulds, all the isolates were sub-cultured on several media (WLN, YGC, DG18, MEA, CYA, and PDA).

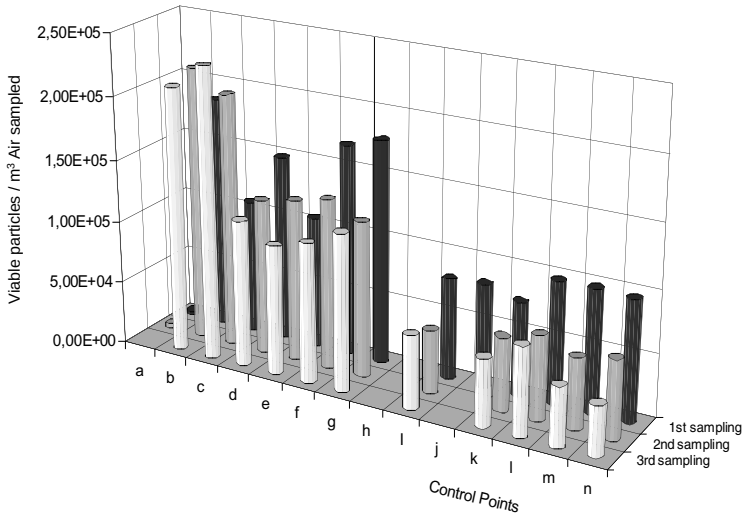
Extraction and quantification of moulds from cork samples. Cork samples were collected in each step shown in Fig. 1, whenever possible. The extraction of moulds from different cork samples was performed using the methodology recommended by the Portuguese Institute for Quality (IPQ) NP 3725 with modifications (Anon. 1989). The method consists of removing micro-organisms from cork samples using 200 ml of a sterile solution of malt extract (17 g l^{-1} in distilled water, pH 4) to which ethanol at 8% (v/v) is added. Incubation is performed at 27 °C with agitation (140 rpm). After 24 h, adequate dilutions of each suspension are performed and used for counting: (I) 10 ml of each suspension sample is sterile filtered through a 0.45 µm membrane (Nuclepore), which is placed on WLN agar. The plates are incubated at 27 °C (Anon. 1989); (II) 0,5 ml of each sample is spread at the surface of the culture medium YGC (Anon. 1998). Incubation of these plates is performed at 25 °C. Colony counting is made every 24 h during 5 days. The results are expressed as the number of viable colonies per weight of cork material in grams (CFUg $cork^{-1}$).

Factory A



Factory B_1 + B_2





Factory C

Fig. 2. Number of viable particles per air sampled in the different sampling points at factories A, B and C, in that order. a) Boiling; b) Sorting and removing the edges of cork slabs; c) Maturing stage; d) Strips cutting; e) Punching; f) Electronic sorting; g) Dimensional correction; h) First manual sorting; i) Washing; j) Drying; k) Final electronic sorting; l) Final manual sorting; m) Second manual sorting; n) Final treatment and packing.

Identification of moulds

The isolates were identified to the genus level based on colony characteristics and microscopic observation by the procedure of slide culture (Larone 1987). The species *Chrysonilia sitophila* was identified according to Pitt and Hocking (1997).

Results

Airborne spore assessment

The results obtained by air sampling procedures, in the four factories and in the different sampling visits to each factory, are summarised in Fig. 2. Factory A shows uniform numbers of colonies in all sampling visits – 1st sampling (May), 2nd sampling (June), 3rd sampling (January) and 4th sampling (March), in each step sampled. In the first step of the manufacturing process, which is performed in the open air, the number of colonies is very low compared to the values obtained for the rest of the steps, which represent indoor areas of the factory.

Factories B_1 and B_2 were sampled in June (1st sampling), in November (2nd sampling), in February (3rd sampling) and in April (4th sampling). In factory B_1 (Fig. 2) airborne contamination was higher in the first two visits. Factory B_2 has very uniform numbers of colonies in all four sampling visits, for all the areas analysed. As already mentioned for factory A, the first step registers the lowest number of colonies sampled.

The air was sampled three times in factory C, in July (1st sampling), in October (2nd sampling) and in March (3rd sampling). The results obtained are uniform along the year, for each area sampled. The lowest number of viable particle counted corresponds to the first manufacturing step, which is performed in the open air, as already mentioned.

Mould diversity on cork slabs during the maturing stage

Considering all the steps in a cork stopper factory (Fig. 1), fungal development is more intense during the slabs maturing stage. The methods utilized were the direct isolation of moulds and its subsequent identification (see Materials and Methods). The subsequent steps depend upon this colonization. Therefore, mould diversity was only assessed during this phase of the cork stopper manufacturing process. *C. sitophila* was always the dominant mould isolated from cork slabs during the maturing stage. However other moulds were also found in the samples brought to the laboratory, namely *Mucor* sp., *Penicillium* sp. and *Trichoderma* sp. and also some *Aspergillus* and *Cladosporium*.

Mould colonization along the manufacturing process of cork stoppers

The results of the microbiological analysis obtained from each type of cork sample are shown in Fig. 3. These results were obtained by extraction and quantification of moulds from cork samples (see Materials and Methods). Due to their higher contamination, moulds counting from cork samples ahead of stopper punching were performed using technique I (see Materials and Methods), while those of the other samples were performed using technique II (see materials and methods).

Discussion

The differences in the manufacturing strategy used by the four factories explains the differences in the numbers of viable particles m^{-3} of sampled air collected throughout the cork stopper processing. The main differences between factories are: (1) in factory A the sorting of slabs and removal of the edges are performed before the maturation step; (2) factory B is formed by two completely separated plants and (3) in factory C punching and dimensional correction of the cork stoppers occur side by side in the same manufacturing area (Fig. 1).

The higher concentration in airborne particles in all the factories sampled was observed in the areas where edges are removed from the cork slabs and during the maturing stage (Fig. 2). This is not surprising, as the first operation creates dust and during the second, heavy fungal growth is obtained. Nevertheless, factory A, in global terms, presents the lowest levels of contamination in those steps of the process (Fig. 2). This factory showed all but 8 sampled points lower than 5×10^4 viable particles m^{-3} of sampled air while the other factories only revealed comparable values after the cork stoppers washing places. This can be due to the fact that in factory A the edges from cork slabs are removed before the maturing stage. Slabs are therefore cut at higher humidity and before mould growth occurs, thus avoiding dust formation. Factory B (Fig. 2), which also presents relatively low levels of contamination, has the operations of sorting, first maturing stage, and removing edges geographically separated from the rest of the operations. This is also a good solution to lower environmental contamination if we compare the results with Factory C (Fig. 2) in which maturing stage and removing edges from cork slabs occur side by side in the same manufacturing area.

After the dimensional correction of the cork stoppers, the viable particle counting are not very different from factory to factory. These operations do not produce as much dust as the ones described before.

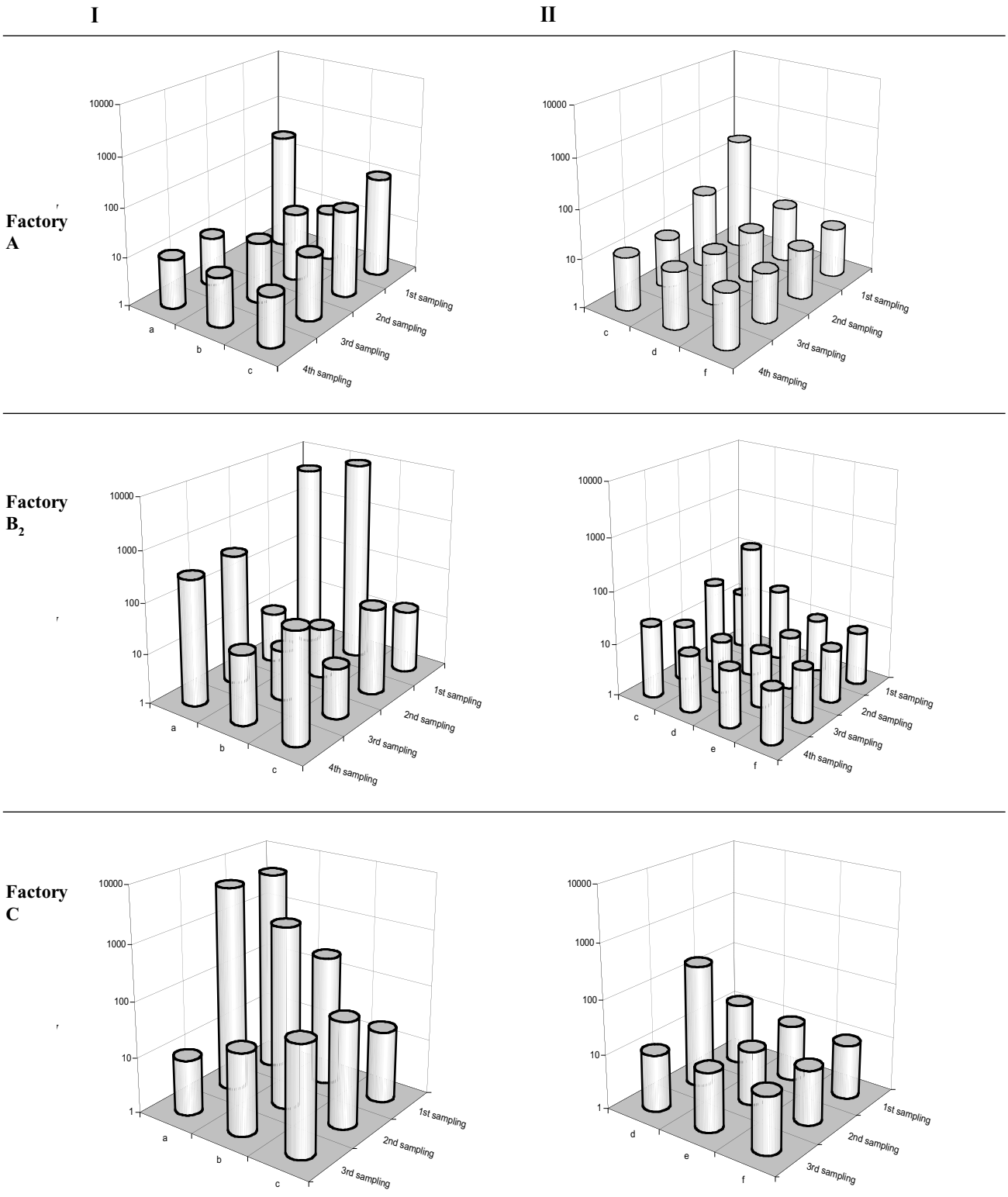


Fig. 3. Mould colonization in the different factories using technique I [punched slabs (a), punched stoppers (b), dimensionally corrected stoppers (c)] and using technique II [(c), washed/dried stoppers (d), waterproofed stoppers (e), packed stoppers (f)]. In order to distinguish zero counting from not determined ones, all counting were added by 10 units, so the value 10 in the graphs correspond to 0 CFU

The extremely low viable particle counting in all the boiling areas are due to the fact that the operation occurs outside the manufacturing pavilions, and stresses the differences in numbers inside and outside the buildings, as already mentioned.

No significant differences in results were observed in seasonal sampling. The differences observed in factory B_2 in the first two visits compared to the third and fourth ones can be attributed to an unusual movement inside the factory due to unpacking of highly contaminated cork slabs packages.

The results presented in this work are only indicative of the air quality in this type of industry, as no legal limits exist for occupational exposition to biological dust particles. Nevertheless the environmental hygienic conditions can be improved by the installation of ventilation systems and aspiration equipment. The use of personal protection equipment may be restricted to the areas where dust accumulation is consistently higher (see Fig. 2).

The mould diversity analysis on cork slabs during the maturing stage revealed *C. sitophila* as the dominant mould, as stated earlier. This is in agreement with the results of a previous work where *C. sitophila* was reported as the predominant mould identified throughout the manufacturing process of cork stoppers (Danesh *et al.* 1997). As the growth rate of *C. sitophila* is higher than that of these other moulds, they were only macroscopically observed on dryer slabs having been submitted to longer maturation periods, which confirms previously published results (Silva Pereira *et al.* 2000).

Regarding the diversity of mould species detected during the manufacturing process, the number of different species isolated was higher in factory C. The results are not surprising since slab cutting is done at least three weeks after boiling. Moreover, in factory C, both steps 'slab maturation' and 'removing edges' are performed in the same place, which facilitates spores dissemination and increased contamination.

The mould colonization analysis along the manufacturing process of cork stoppers showed a drastic reduction in CFU numbers throughout the process. If only the final steps are considered, no major differences were observed in the number of CFU of moulds among the industries studied. This reduction was expected due to the continuous reduction of the humidity content of the cork material and the type of final treatments used on the cork material, which introduces a barrier against liquid absorption.

Detailed study of fungal genera that were isolated showed that *C. sitophila* was almost never detected after the punching of the cork stoppers. Some *Penicillium* sp. were still isolated on the cork stopper samples throughout steps of cork washing and sorting. It is possible that this genus, contrary to what happens to *C. sitophila*, still maintains viability in the finishing steps of the stopper process, although water activity has been much reduced.

In factory B_1 *C. sitophila* was the dominant mould isolated from cork slabs. Along the manufacturing process a reduction of moulds occurrence was observed and *C. sitophila* was never detected after the punching of the cork stoppers. These results may be due to the complete separation of boiling and maturing steps from the subsequent processing steps by two separated plants (B_1 and B_2).

The moulds isolated and identified in the slabs of factory C were *Penicillium* sp., *Aspergillus* sp., *Trichoderma* sp., and *C. sitophila*; however, this mould was not always the predominant one.

Silva Pereira *et al.* (2000) reported the ability of *C. sitophila* to restrict the growth of other moulds over maturing slabs for a period of 30 days. These authors also concluded that *C. sitophila* does not produce 2,4,6-trichloroanisole (TCA), guaiacol, or 1-octeno-3-ol during its development on cork slabs, and is able to metabolise clorophenolics without producing significant amounts of TCA. Accordingly, the above reported results justify the interest of the producers to develop sanitary strategies that favour the growth of *C. sitophila* on the maturing cork slabs in order to reduce the colonization by opportunistic moulds, for example *Penicillium* sp. On the other hand, it can be expected that the development of *C. sitophila* may be less aggressive for the workers health as *C. sitophila* spores (6–15 μm) are bigger than those of *Penicillium* (3–3,5 μm). In fact, *C. sitophila* spores enter only in the airways of the respiratory system, and the lung lesions (asthma) that they can cause are reversible if workers are moved to a different area, while *Penicillium* spores can reach the lower part of the respiratory system, penetrating beyond the terminal bronchioles into the gas exchange region of the lungs. This can cause severe and usually irreversible lung lesions (suberosis) (L. Delgado, personal communication).

In factory C, the slabs contamination at the stage of 'removing edges' was higher than in the other factories (Fig. 3). The results are attributed to the configuration of the installations and processing. The number of CFU of moulds decreased along the cork stoppers manufacturing processes, as in the other factories.

It must be emphasised that in all the factories reported here, finished stoppers always presented good microbiological quality (ΣCFU/cork stopper ≤ 4).

According to the results reported above, it can be concluded that the spatial separation of the different phases of the process, including physical separation of the maturation phase, is convenient to guarantee high air quality and appropriate cork slabs colonization. The

sorting and cutting of the edges of cork slabs after boiling and before the maturing step, instead of the usual procedure, is also highly recommended.

Acknowledgements

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