Application of Luminescent ATP Rapid Checks at Ready-to-eat Foods Producing Plant

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Abstract

Caputo P., Ferri E.N., Girotti S., Gozzi S., Saracino P. (2011): **Application of luminescent ATP rapid checks at ready-to-eat foods producing plant**. Czech J. Food Sci., **29**: 382–390.

Bioluminescent ATP determination has been applied at a ready-to-eat meals producer plant as a screening tool to evaluate the cleaning procedures and identify possible sources of microbial contamination. Luminescent ATP test revealed the risk situations in few minutes, any time it was supposed useful, and on site. All data were confirmed by the plate count method and immediate interventions were undertaken in risk situations, concerning not perfectly cleaned surfaces and cross-contamination between the areas for different types of foods. Various departments have been checked: cold stores, raw food manipulation areas, kitchens, coolers, and packaging areas. Critical situations were revealed in the fresh foods cold storage areas, which were consequently treated by more intensive cleaning procedures, and the raw food manipulation areas, which were physically separated by walls, were more intensively cleaned with exchanges between the various areas strictly regulated. Intervention effectiveness was confirmed by repeated controls using portable ATP assay, since the evaluation of the relationship between CFU and RLU gave good results, with an r^2 value of 0.83.

Keywords: bioluminescent ATP assay; ready-to-eat meals; hygiene conditions; rapid screenings

In developed countries, the consumers are taking unprecedented interest in the way food is produced, processed, and marketed and they are increasingly calling to food processing enterprises and retailers for complete and documented guarantees on the quality and safety of their products. In determining the overall quality of the packaged ready-to-eat foods, to use selected materials and to operate in optimal hygiene conditions are equally important parameters. Proper cleaning and effective sanitisation activities contribute significantly to the prevention of the products contamination by microorganisms causing food-borne diseases or spoilage. On the other hand, the process control systems and integrated quality and safety assurance programmes implemented in food industries rely on the idea that safety and quality of the products are best controlled through effective management of those processing areas where hazards may arise. All activities to be performed by manufacturers to ensure high quality of their products can be identified with careful application of the Good Manufacturing and Hygiene Practices and the identification and control of the critical phases of the production processes according to the HACCP rules (US FDA 1997, 2004; European Commission 2005). The HACCP system approach to the management of the microbial hazard suffers from slow and cumbersome conventional methods in microbiology which allow neither a rapid evalua-

Supported by the University of Bologna "Focused Fundamental Research Projects 2008–2009".

tion of raw materials nor real-time monitoring of the measures taken during processing. The end-product tests often permit only a retrospective assessment of microbiological conditions. The fundamental information, especially on pathogens, offered by the microbiological tests, cannot surely be disregarded but the application of the methods which enable more rapid and less expensive estimation of microbial contamination on the several critical points in a processing plant is of great help for timely interventions.

Rapid assays to monitor the presence of organic residues on the surfaces and plant components are equally important, since the elimination of the potential proliferation substrates helps to ensure a complete and prolonged effect of the sanitisation procedures: the amount of organic material greatly influences the vitality of micro-organisms (De Cesare 2003) as well as the creation of biofilms on the surfaces, with increased difficulties in removing them (Shi & Zhu 2009). This specific aspect cannot be in any case revealed by the classical microbiological plate counts.

Rapid tests like the ATP (adenosine triphosphate) methods can be used to reveal the presence of both somatic cells (like food residues) and microbial contamination, obviously not distinguished by these tests.

The luminescent ATP assay employs the light-producing firefly luciferin-luciferase system to quantify the ATP of any origin, released by bacterial and/or somatic cells (Girotti *et al.* 2001, 2003); it has been, and it still often is, employed to assess the hygiene conditions of the surfaces and air in different situation, including food processing or manipulation (Aycicek *et al.* 2006; Romanova *et al.* 2007; Whitehead *et al.* 2008). It requires few minutes to give a reliable assessment of total biological contamination, a parameter which has already been demonstrated to offer information on the hygiene condition of a surface comparable to those obtained from the aerobic plate count test (Caputo *et al.* 2008; Vilar *et al.* 2008). Being easy to carry out and cheap, this assay does not identify pathogenic bacteria but can be especially useful as a rapid screening tool where the risk of contamination is believed high, when a continuous monitoring is requested to maintain the highest quality levels, or where low cost controls are required for economic reasons.

We applied this assay to obtain a rapid evaluation of the presence of micro-organisms and/or food residues on food contact surfaces and equipments in a producing plant of an Italian company leader in the preparation of ready-to-eat meals consumed daily at canteens, refectories, fast-food shops etc, or packaged under modified atmosphere (MAP), and distributed mainly to supermarkets.

The monitoring of microbial contamination and the assessment of microbiological quality of the final products including the identification of the food pathogens were done usually by aerobic plate count and specific cultural or analytical methods. Because of the urgency to perform at a reasonable cost and in the shortest time possible a particularly detailed evaluation of hygiene conditions in all areas and their evolution during the production process and during the week-end, the use of a rapid assay was requested. Its reliability in this specific investigation was confirmed by the aerobic plate count method on a significant number of samples.

For example, the evolution of the contamination levels during the week-end was followed, focusing the attention on the Monday morning conditions before the re-start of the production, the tests being carried out without a new cleaning treatment. It was expected that during the week-end microbial proliferation or contamination could occur. Any occurrence of cross-contamination between different areas was another crucial aspect to be investigated.

Moreover, the microbial content in air, the microclimatic parameters, and the air quality defined as the presence and quantity of gases (namely CO_2) in a composition different from fresh air were under investigation. All these parameters usually employed to predict the indoor comfort feeling must be also taken into account to ensure good overall hygiene conditions in the production plants (FAO/WHO 2003).

MATERIAL AND METHODS

Standard cleaning procedures. Cleaning and disinfection of benches, rooms, and equipment were done daily by using suitable detergents and antibacterial solutions supplied by Ecolab S.p.A., (Milan, Italy) if not indicated otherwise. The same products were employed in the integrated cleaning systems of equipment such as the self-cleaning ovens, as well as when manual procedures were required. In the area for raw food manipulation and in the kitchen, a simple cleaning, without sanitisa-

tion, was done on the benches surfaces after each preparation, i.e. various times per day.

– Inside the cold stores, the stand surfaces were cleaned before the storage of each supply, the walls once a month.

– In the kitchens, the tests were performed in particular on meat frying pans, self-cleaning ovens, and coolers.

– In the department for packaging of cooked foods under modified atmosphere (i.e. 30% CO_2 70% N_2) (MAP), apart from the usual sanitisation procedures, the scales for portions weighing were cleaned manually during work each 15–20 weighings, to avoid the presence of food debris. The coolers for the cooked foods were analysed at each sampling session.

Sampling. Both for aerobic plate count and for bioluminescent assay, a collection of three samples at each sampling point was taken in parallel, twice per each working day: in the morning before the opening of the plant, and in the evening following the final cleanup procedure, with the aim to intercept any contamination which could occur during the day (Powell 2006).

Microbiological plate count. To perform the aerobic plate count, RODAC plates (Liofilchem s.r.l., Roseto Degli Abruzzi, Italy) of 6 cm diameter containing non selective agar medium or a chloramphenicol-added medium were put into contact for 1 min with the surfaces. The first kind of plates were incubated at 37°C for 24–48 h, to allow the growth of bacteria; the second one with chloramphenicol to select moulds and yeasts, for 48–72 hours. The values indicated as microbiological counts corresponded to the sum of the numbers of colonies developed on both media. A very low threshold value for the plate counts (Colony Forming Units – CFU) to be respected in the production and packaging of food was chosen: 30 CFU, when usually a threshold value of 50 is accepted by the US and EU directives mentioned above. Counts 15 units higher than the threshold value defined a risk of contamination because of the possible bacterial proliferation after a certain time, while counts of 60 CFU higher identified not sanitised areas with a high risk of bacterial contamination and bacterial growth.

Bioluminescence ATP assay. Each sample was collected by swabbing an area of 10 × 10 cm by using the UltraSnap swabs (Hygiena International, Watford, UK). The swab was introduced in a capped tube containing all reagents for microbial ATP determination, i.e. the extracting solution to dissolve the cells and obtain free ATP and the luciferin-luciferase detection system. Once the cap had been opened and the reagents had come into contact with the sample, the tube was shaken vigorously and introduced into the portable luminometer "System SURE II" (Hygiena International, Watford, UK) to measure the emitted light, directly proportional to the ATP extracted from all cells, bacterial or not, vital or not, present in the sample.

The results of these measurements were reported as Relative Luminescence Units (RLU) expressing the light intensity, which could differ for the same sample depending on the sensitivity of the specific instrument. In the same way, the threshold value indicating a sanitised surface is different for each instrument and is determined by the supplier according to the calibration experiments performed during the development and optimisation of its device. In our case, it was fixed at 50 RLU. Values 15 units higher than the threshold define a risk of contamination because of bacterial proliferation after a certain time, while values 100 RLU higher identify not sanitised areas, with a high risk of bacterial growth (Hygiena 2009). All surfaces and devices were usually rinsed after the manual or automatic cleaning; nevertheless, any possible interference by the product residues in the bioluminescent emission tests was evaluated by adding to the luminescent system various dilutions of the cleaning products and aliquots of a 0.2mM ATP solution instead of the real sample extracts.

RLU values and CFU counts were plotted as they were obtained or as log 10 RLU and log 10 CFU. Linear regression analysis was carried out to evaluate the relationship between the two measures.

Assessment of the microbial content in air. The amount of aero-dispersed micro-organisms was determined by an Air Sampler SAS-Super 100, supplied by PBI (Milano, Italy), with an air flow of 100 l/min and a sampling time of 500 s, employing the same RODAC plates as used for the surfaces sampling (both types). The quantity of micro organisms dispersed in air was determined by the plate count as described above for the surfaces test, following the same incubation conditions.

Microclimatic parameters and CO_2 concentration. The dry and wet bulb temperatures and the globe temperature were the microclimatic parameters taken into account. To collect these data, a "Thermal Environment Monitor QUESTemp 36",

integrated to an anemometer AIR PROBE-S, both from QUEST Technologies (P.C. Werth Ltd. London, UK), was used. The measurements lasted 10 min and the instrument was connected to a computer for the data processing.

CO_2 concentration was measured by a Q-Check TM CO_2 Meter 8731, supplied by the TSI Incorporated (Shoreview, USA). This instrument was equipped with a NDIR (Non Dispersive Infra Red) detector and CO_2 concentration was expressed in ppm. The measured values both for microclimatic parameters and CO_2 concentration were compared to the values usually reported to indicate good indoor conditions (British Standard Institution 1994, 2006; US EPA 2009).

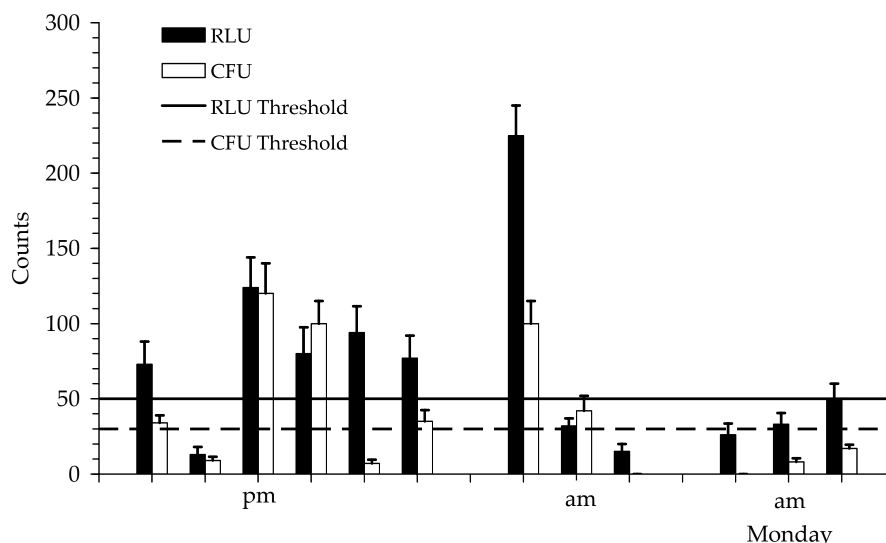
RESULTS AND DISCUSSION

The tests performed to assess the influence of the cleaning product residues on the ATP light emission showed that no stimulation effect by any of the products employed was detected, ensuring that the recorded light was produced only by the extracted ATP. At 10^{-3} molar concentrations, different for each product, the inhibition of light emission was observed, but it was unlikely that such quantity remained on the surfaces rinsed after the treatments. Moreover, the good agreement between the plate count and luminescent data obtained during all monitoring activities allowed affirming that the possible residues of the cleaning products on the surfaces were not present in sufficient amounts to interfere with the bioluminescent assay.

As expected, the major part of specimens indicated that the correct hygiene and sanitary measures had been followed. The few situations in which a microbiological risk was detected are discussed here together with the actions taken to eliminate the risk.

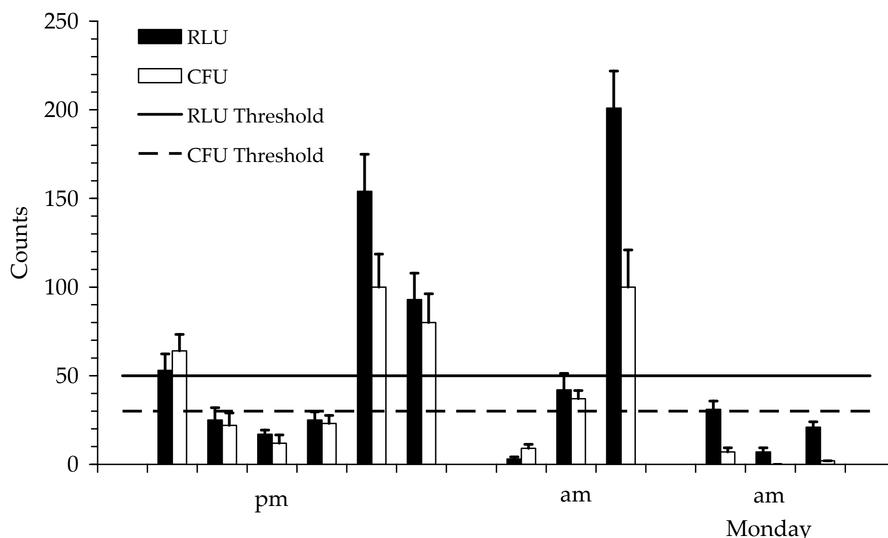
In particular, in the cold storage area for salt pork and cheeses, the luminescent and plate count data from the samples collected on the walls were always under the threshold value, but about 50% of those from the stand surfaces were above this value, reaching plate counts up to 100 CFU and light emission values above 300 RLU. Concerning the area for fresh vegetable cold storage, 50% of the samples collected both on walls and stand surfaces showed the counts up to 100 CFU. In this case, the luminescent assay reached values above 800–1100 RLU, showing that the most important contamination had come from organic residues.

These data were not surprising because this was the storage area for the products directly coming from the market, before washing or any other treatment. The plate counts and ATP assay were performed on the samples from this area to get an idea of the microbial contamination produced



am – samples collected in the morning before starting the production; pm – samples collected in the evening after the final cleaning and sanitisation; am Monday – samples collected Monday morning before starting the production; CFU threshold value 30; RLU threshold value 50

Figure 1. Total plate counts (CFU) and ATP-dependent light emissions (expressed as RLU) obtained for the samples collected in the area for raw vegetable manipulation



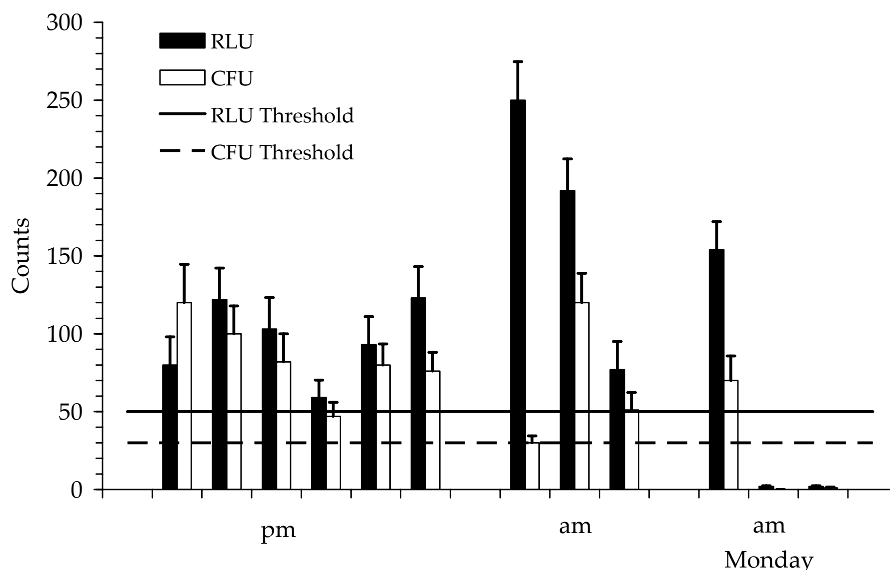
For explanation am, pm and am Monday see Figure 1; CFU threshold value 30; RLU threshold value 50

Figure 2. Total plate counts (CFU) and ATP-dependent light emissions (RLU) obtained for the samples collected in the area for raw meat manipulation

by these raw materials. High contamination in the areas for fresh products was in any case undesired and, on the basis of the results, the supplier of the cleaning products, also responsible for compliance with the regulatory rules for all cleaning procedures, decided to increase the final concentration of the detergent/disinfectant solutions to be used in these areas. The 1:20 or 1:30 dilutions were applied instead of the previously used 1:100 dilution. The ATP assays were repeated, revealing that this change produced a better hygiene situation in the area for fresh products, reducing the total level of organic contamination: the maximum RLU values were in the range of 500–700.

Another department in which critical situations were identified was that for raw food manipulation. The results obtained for a group of samples concerning both the aerobic plate count and the bioluminescent assay and collected in the areas for vegetable, meat, and cheeses manipulation are showed in Figures 1–3.

Taking into account all samples from the above mentioned areas, independently of the moment of sampling (morning or evening), about 55% reached



For explanation am, pm and am Monday see Figure 1; CFU threshold value 30; RLU threshold value 50

Figure 3. Total plate counts (CFU) and ATP-dependent light emissions (RLU) obtained for the samples collected in the area for raw cheeses manipulation

or surpassed the respective threshold values. A more noticeable situation was that observed with the samples collected in the evening following the final cleanup: 60% of these samples showed a CFU number indicating not sanitised situation, while 78% produced RLU emission above the threshold value, suggesting that not negligible amounts of organic residues were present. In the morning before opening, the CFU values of about 67% of samples and the light emission of 55% of them were above the respective threshold values. Of course, the daily cleaning of the benches in this area had been defined as not satisfactory, especially in the area of cheeses manipulation, in which also the level of the organic residues resulted as particularly high (Figure 3).

As a consequence, it was decided firstly to employ also in this department the more concentrated solutions of the cleaning products. At the same time, important changes were designed involving the wall structures between the areas dedicated to different kinds of foods as well as new rules concerning the work clothes and free movement of people around the various rooms. First of all, each area dedicated to the manipulation of a specific food (meat, vegetable, fish, cheese, etc.) was completely separated by walls from all others. The possibility to move freely from one area to others, as it was before, was excluded. The unique access to each area was a door, kept shut. The personnel working in each area were strictly assigned to it, and in case it was necessary for a person to move from one room to another, this could occur only after wearing new, clean work clothes. In this way, the possibility of cross contamination between foods with different microbial contents was completely excluded.

The ATP assay, repeated after changing the cleaning solutions and the rearrangement of the various areas, confirmed the effectiveness of the interventions taken. A reduced number of samples, about 20% of those collected in the area for cheeses manipulation, revealed the persistence of some organic residues showing values in the range of 100–150 RLU.

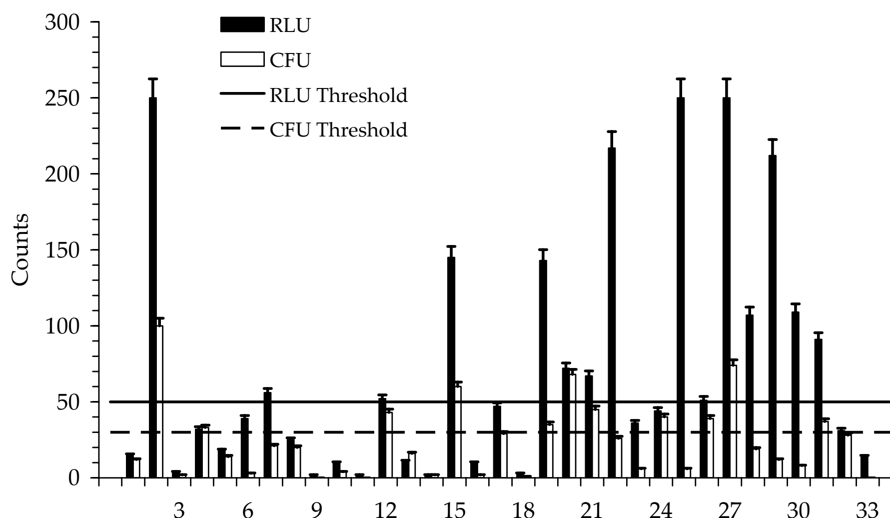
On comparison between all samples collected during Friday evenings and those collected on Monday mornings before the start of the production it was noticed that, surprisingly, only 17% of the samples collected showed values above the admitted CFU or RLU limits. An increase in microbial contamination was expected after the week-end stop of the activities, but this was not the case. Probably the prolonged drying of surfaces did not allow the survival of micro-organisms which remained still alive immediately after the evening cleaning.

All equipment and surfaces in the kitchens department resulted as being free of bacterial contamination and organic residues, with the only exception of the cooler: 10% of the samples from this device were indicated as "not sanitised" by both tests. The problem was presented by the not ideal position of the cooler, placed too far from the pump dispensing the detergents solution under pressure: an easy access of the automatic cleaning device to the inner surfaces was not possible. This problem was immediately solved by distributing the cleaning products inside the cooler directly by hand, thus obtaining the reduction of the plate count to zero.

The hygiene conditions of the kitchenware were evaluated by testing each time a large number of pieces and collecting the data independently of the day or the moment of sampling during the day. A representative group of data obtained from both assays is showed in Figure 4. It can be seen that the situation was quite critical, concerning the contamination by both food residues and micro-organisms.

The kitchenware were all washed in dishwashing machines using suitable detergents, but the washed kitchenware stood, not covered, on shelves cleaned once at week; the presence of food residues and bacteria was judged a risk situation, which required some changes. The kitchenware showing grooves or other damage making a precise cleaning difficult were disposed off; a stronger rinsing flow was obtained by substituting a component of the dishwashing machine, and the water temperature was set at higher values. Moreover, the less frequently used kitchenware were separated and placed in a closed stand. The RLU values measured on kitchenware randomly collected after these changes showed a significant decrease of the number of samples occurring above the threshold value: from above 40% to 25%.

The department dedicated to the modified atmosphere packaging of foods was probably the most important to test: re-contamination of cooked foods by pathogens such as *Listeria* sp., able to grow at the refrigeration temperature, surely represents a serious threat to the packaged food safety. The samples were collected from the surfaces which



CFU threshold value 30; RLU threshold value 50

Figure 4. Total plate counts (CFU) and ATP-dependent light emissions (RLU) obtained for a group of kitchenware.

could be in contact with cooked foods, the coolers, and the scales for weighing the portions. All samples were completely negative for the presence of both food residues and bacteria, confirming a perfect cleaning.

The microbial content in the air in the different departments changed according to the activities carried out and to the number of people working there but the plate count never showed values which could represent a hygiene risk for the food products. For example, the total plate counts for the samples collected at the department for modified atmosphere packaging showed a number of colonies similar to that detected in a surgery room, confirming the perfect sanitary situation. On the contrary, about one third of the samples collected in the kitchens developed a number of colonies which was just above the threshold value. This situation, already described as common during the meals preparation (Haysom & Sharp 2005), was explained by the continuous introduction of a lot of not cooked materials and personnel movement through the room and was not classified as a risk situation.

The general good hygiene conditions in the whole building were also confirmed by the satisfying val-

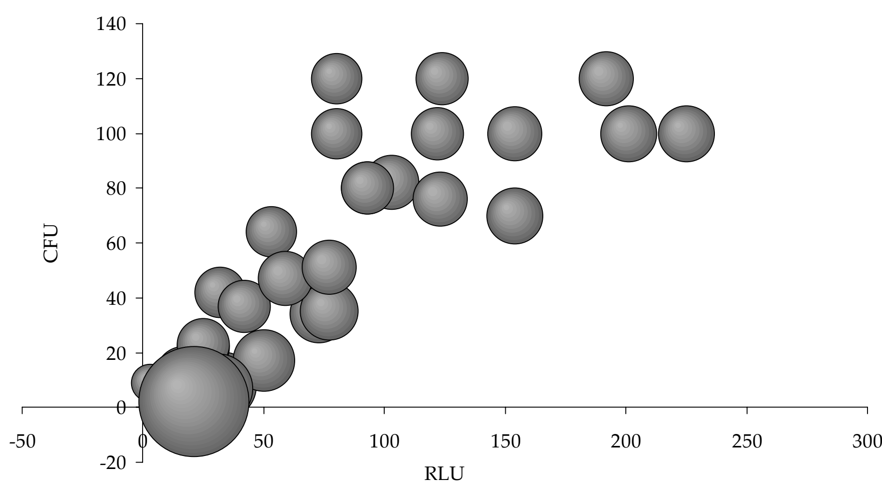


Figure 5. Correlation between the CFU and RLU values. The spot size is proportional to the frequency of the corresponding values. The wider distribution of the higher RLU values can be ascribed to the contribution of the somatic ATP (organic residues)

ues of the Fanger Indices (PPD and PMV) (BRITISH Standard Institution 2006) calculated on the microclimatic parameters (temperature, humidity, radiant temperature) and by the CO_2 levels. The value of 400 ppm was rarely overcome indicating that the air replacement was adequate: the values indicating stale air are usually fixed above 1000 ppm for working places (US EPA 2009; Air and Energy Services Inc.. 2003).

The aim of the work, i.e. to evaluate the reliability of rapid assays in obtaining a timely but careful picture of the sanitary conditions in various points of the plant, was fully attained. Taking into account that the CFU counts indicate only the viable bacteria while the RLU counts include both bacterial and somatic ATP, the correlation (log RLU vs log CFU) calculated for the two sets of data, expressed by the equation: $y = 1.0272x - 0.2232$, with a $R^2 = 0.83$, can be considered very good. In Figure 5, the slightly wider distribution of RLU values is evident.

The application of the luminescent ATP assay allowed to identify accurately any weak point in the cleaning of the plant surfaces and devices, as well as to verify immediately and step by step the effectiveness of the changes introduced in the cleaning protocol or in the spatial distribution of various activities. The useful results in terms of the final products quality, described here, were obtained supporting counts about one third with respect to the plate count method, with the possibility of entrusting the cleaning personnel with the performance of these simple measurements. This study was the umpteenth demonstration that rapid assays like the ATP luminescent one can play an important role in a regular, effective checking of the actual conditions in food processing plants to ensure high quality and safety of the final products.

Acknowledgements. The authors acknowledge Ecolab S.p.A. for the strict collaboration in optimising the cleaning products formulations according to the investigation outcomes and Mr. Pietro Tabegna for his precious support during the sampling activities.

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Received for publication May 26, 2010
Accepted after corrections October 18, 2010

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