Evaluation of ATP-bioluminescence based methods for hygienic assessment in fish industry

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Running headline: ATP measurement in fish industry

Abstract

Aims: To evaluate ATP bioluminescence-based hygiene monitoring systems under conditions relevant for fish processing environments.

Methods and Results: The ATP bioluminescence of fish fractions that are potentially present after insufficient cleaning of fish processing environments was determined. Different fractions and interfering substances representing the stages from slaughtering to smoking were prepared and measured using two different commercial systems. ATP bioluminescence was quenched by acidic liquid smoke and by sodium chloride even at concentrations as low as 0.9% NaCl. Large variations were observed between different types of trout homogenates: The ATP bioluminescence from raw belly fat homogenate were 100-1000 timer lower than for trout blood. There were about a 1000-fold lower ATP bioluminescence in raw, compared to heat treated fractions from trout, with the exception of blood. The bioluminescence from *Listeria monocytogenes* was very low. Results from fish processing plants supported the laboratory findings.

Conclusions: The output from ATP-monitoring instruments depends on the nature of fish soil present, as well as the presence of sodium chloride and low pH. This may lead to considerable under- or overestimation of the level of organic soil.

Significance and Impact of the Study: ATP bioluminescence instruments are widely used by the fish industry for monitoring hygiene. The monitoring method will only give valuable information about the hygiene if critical limits are set after a validation period, distinguishing between areas with different type of soil and between different hygiene zones.

Keywords: ATP; hygiene; food processing; quality control; microbial contamination

Introduction

Fresh fish is a perishable type of food and strict control of storage conditions and hygiene are prerequisites for production of products with a stable and good sensory quality. Reducing the incidences of exposing consumers to products with poor microbial quality could potentially reduce a barrier for eating fish. During processing, fish residues may accumulate in the processing environment and result in growth of microorganisms. Such microorganisms can again be transferred to fish products through cross-contamination and impact food safety and food quality (Møretrø and Langsrud 2017). As it may be difficult to eradicate bacteria established in the processing environment, e.g. as biofilms, the emphasis should be on to preventing establishing of bacteria. This can be obtained through proper hygienic design, efficient cleaning and disinfection and a hygiene monitoring program that discovers possible threats for the quality early rather than collecting historical data. Thus, there is a need for accurate and fast hygiene monitoring methods that can measure the level of residual soil and microorganisms after sanitation, to be able to initiate corrective actions before bacteria have established themselves in the processing environment.

Many processing plants use a considerable amount of resources on monitoring the cleanliness of the processing environment by sampling and analyzing for total bacteria by conventional cultivation dependent methods (Møretrø and Langsrud 2017). Plants producing ready to eat products like cold smoked salmon or sushi may also analyze for the pathogenic bacteria *Listeria (L.) monocytogenes*. Disadvantages with cultivation dependent methods are that they are relative expensive and that it usually take several days to get the results, thus it is difficult to use the results dynamically in order to improve the cleanliness of surfaces as part of the HACCP system (Hawronskyj and Holah 1997). A long response time may also increase the risk of bacteria establishing themselves in the processing plants and also the risk of products with low quality and increased safety risk reaching the market before the results are available.

Easy and fast alternatives or supplements to bacterial cultivation based monitoring methods are chemical or enzymatical methodologies based on monitoring residual soil after cleaning and disinfection. Monitoring of the hygienic level of surfaces by measuring bioluminescence as a function of the ATP concentration is increasingly popular in the food industry (Davidson et al. 1999; Dostalek and Branyik 2005) as well as in hospital settings (Amodio and Dino 2014). There are many different ATP measuring systems at the market, but their common principle is based on that all living organisms contain ATP and that the enzyme luciferase as a function of the ATP concentration will emit light which can be measured as relative light units (RLU) with a luminometer (Hawronskyj and Holah 1997; Champiat et al. 2001). Such methods are providing results within minutes and are considerably cheaper compared to cultivation dependent analysis of total bacteria or pathogens like *L. monocytogenes*.

In the light of the popularity of the ATP-luminometers it is surprisingly little documentation on the relation between the amount of food residues and number of RLU measured and the robustness of the assay to environmental factors. Food industries differ substantially with regard to the sanitation regime, type and amount of soiling and type and amount of microorganisms present. Also, large variations occur within the same processing unit, for example between a slaughtering line (blood) and ready product (cooked product). It is reported that the measured bioluminescence from different types of food varies up to 1000 times (Corbitt et al. 2000; Whitehead et al. 2008; Viator et al. 2017), but to our knowledge the relation between the amount of different types of complex residues that can be found at different stages of processing and ATP bioluminescence has not been investigated. Secondly, it has not been reported how processing ingredients like salt as well as common processing steps like heating and smoking affect the level of ATP measured by the bioluminescence method. Finally, there is limited information available on ATP content of bacteria dominating in fish processing environments. The ATP method is unspecific, as all living cells contain ATP, and both soiling from food residues and bacteria will contribute to the signal.

Knowing about the differences in measurable ATP-levels in fish fractions and the influence of processing conditions and common fish associated bacteria on ATP measurements is a starting point for translation of ATP readings to a metric for the hygienic level. This will enable establishment of appropriate critical limits for taking corrective actions, and thus improve the quality of decisions made from such results.

The aim of this study was to monitor the ATP-content, as measured by two commercial instruments based on bioluminescence, of different variants of fish residues that will be found along the same processing line from slaughter (blood), gutting (fat rich), filleting (protein rich) and heating (cooked fish), and the effect of processing contaminants from the smoking process (salt, liquid smoke). Furthermore, hypotheses derived from the laboratory work were evaluated based on sampling/data from three fish factories.

Materials and Methods

ATP instruments and tests

Several hand held ATP measurement devices are on the market. We choose to use the SystemSURE plus™ (Hygiena, Camarillo, CA, USA), as this instrument/luminometer is commonly used in the Norwegian food industry and was already in routine use by two processing plants involved in the project associated with the present study. SystemSURE plus was used with UltraSnap ATP sampling swabs (Hygiena). In addition, the 3M Clean-Trace NG Luminometer with Clean-Trace surface ATP UXL100 swabs (3M, Hammfelddamm, Germany) were used. This instrument was routinely used in one processing plant involved in the project associated with the present study.

Preparation of muscle/blood samples

Different types of fish residues may be present along the processing lines after cleaning. Various fish samples were prepared and analysed for ATP and chemical content. Farmed trout on ice and blood collected from the slaughtering line was received from a Norwegian producer. Trout was the major fish type processed in two of the plants and salmon in the third plant involved in the project associated with this work. Two different types of homogenates were prepared: 1. Protein rich, low fat loin meat (Loin) 2. Fat rich belly meat (Belly fat). For both types, the parts of interest were removed/cut out from the fish and cut into pieces. Equal weight of distilled water was added, followed by treatment in a Stomacher for 30 s. For comparison purposes, homogenates of cod loin and minced beef (both purchased from a supermarket) were made; 50 g was added 50 ml dH_2O and homogenated with a Stomacher for 1 min. The filtrate from the Stomacher bag was further diluted 1:3 with dH_2O, and treated with a Stomacher for two times 1 min. The blood and the homogenates were kept frozen at -20 °C until use. Cooked samples were made by exposure to 80 °C for 30 min in

water followed by cooling to room temperature. All samples were tempered to room temperature before ATP-measurements.

Preparation of bacterial samples

The ATP content of bacteria were examined for five strains of *L. monocytogenes* and 10 strains of other bacteria frequently associated with fish and fish processing. The five *L. monocytogenes* strains were all isolated from Norwegian plants processing farmed salmon and to some extent also farmed trout (Møretrø et al. 2017). *Listeria innocua,* from a conveyor belt in a salmon processing plant (Langsrud et al. 2015) was included for comparison with *L. monocytogenes*, as a representative of other *Listeria* species than *L. monocytogenes*. In addition, nine other bacterial strains were included, all isolated from Norwegian salmon processing plants, and representing the genera *Acinetobacter* (2 strains), *Aeromonas* (2), *Pseudomonas* (2), *Serratia* (2) and *Shewanella* (1). These genera are frequently found in Norwegian salmon processing plants (Langsrud et al. 2015; Møretrø et al. 2016), and in fish processing environments in general (Møretrø and Langsrud 2017). All bacteria were stored at -80 °C.

Bacteria were streaked to tryptic soy agar (TSA, Oxoid, Basingstoke, UK), and incubated at 30 °C. Colonies were inoculated in tubes with tryptic soy broth (TSB, Oxoid), which was incubated at 30 °C with 150 rpm agitation overnight. From the overnight cultures, approximately 1 µl was transferred to 3 ml salmon juice (prepared as previously (Langsrud et al. 2015), with the following modifications; after the autoclaving step the suspension was centrifuged twice (8000 g, 5min) to remove as much particles as possible) and incubated at 12 °C with 150 rpm agitation for 3 days. 1 ml of culture from salmon juice was harvested by centrifugation (8000 g, 5 min) and washed once in 0.85% NaCl, before being resuspended in 1 ml 0.85% NaCl. The resulting suspensions were incubated at 12 °C with 150 rpm agitation for 2 days, before ATP-monitoring. The cell numbers of all suspensions were

151 determined by plating to iron agar, and colony forming units determined after incubation at 25 °C for
152 two days.

153

154 **Chemical analyses**

155 Dry weight, lipid, protein, sodium chloride and pH were determined for all the prepared
156 homogenates as well as fish oil. Dry weight was determined indirectly as 100% - % water content,
157 where the % water content was determined according to NMKL 23 (NMKL 1991). Fat content was
158 determined by NMR (Maran Ultra LF-NMR, Oxford Instruments, Oxfordshire, England*).* pH was
159 measured by a pH meter (PHM210, Radiometer Analytical SAS, Villeurbanne, Cedex, France). The
160 Kjeldahl method was used to measure the protein content. Sodium chloride was determined by using
161 a Corning 926 Chloride analyzer (Corning limited, Essex, England).

162

163 **Effect of sodium chloride, fish oil and liquefied smoke on ATP measurement**

164 The influence of sodium chloride on ATP bioluminescence was tested at different sodium chloride
165 concentrations (0, 0.9, 4.5, 9, 13.5, 18 and 21.6 % (w/v)). The effect of salmon oil (H-oil, Hordafor,
166 Norway), and liquefied smoke (used for smoking fish, Smokez Enviro 24PA, Red Arrow International,
167 Manitowoc, Wisconsin, USA) on ATP bioluminescence were tested. The ATP readings of pure
168 substances were also measured as controls. The experiment was performed in triplicate on different
169 days.

170

171 **ATP Bioluminescence assay**

172 For ATP measurement of fish and beef samples, 10 µl were pipetted to UltraSnap or Clean-Trace
173 swabs. If the ATP readings were outside the linear measurement range (>3000 RLU for UltraSnap and

>100 000 RLU for Clean-Trace, as determined by testing with dilutions of pure ATP, Figure S1), the samples were diluted in dH_2O before a new measurement.

To test whether different compounds present on food processing surfaces inhibited or potentiated RLU-signals, 10 µl of a mixture of ATP (1×10^{-7} M final concentration, resulting in 1×10^{-12} mol ATP applied in total) and the chemical compound (or water as a control) were applied directly to the tips of the ATP swabs and the RLU measured according to the manufacturer's instructions.

The bacterial suspensions were diluted 10 times in dH_2O, before a volume of 10 µl of the resulting suspensions was added to Clean-Trace and UltraSnap ATP swabs, and ATP measured according to the manufacturer's instructions.

ATP measurements and microbial sampling in fish processing plants

Sampling in trout and salmon processing facilities

A plant processing trout and salmon, used ATP measurement (UltraSnap/SystemSURE) of surfaces after cleaning for a five month period. During the same period, the plant also used Hygicult TPC dipslide (Orion Diagnostica) to measure bacteria at surfaces after cleaning.

Sampling in facilities receiving and processing white fish

Two Norwegian factories that received freshly caught white fish dominated by cod, haddock and saithe from fishing vessels, and that produced frozen and salted fish were visited and sampled. The time of the visit was off-season, thus no fresh fish were received at the visiting day, however salted fish were stored in trays and there were some activity in the factories. Samples of surfaces like conveyors, machines, trays and floors were taken for a total of 18 sample sites from the two plants. For each sampling point, a surface area of 25 cm^2 was sampled and the ATP level measured with the Clean-Trace system. Total bacterial count was determined after sampling of 25 cm^2 with sterile swabs

(Mesoft, Mölnycke Health Care AB, Gothenburg, Sweden), moistened with saline (0.85% NaCl), followed by plating to iron agar (Oxoid) and incubation at 15 °C.

Statistics

Statistical tests were calculated in Minitab vs18 (Minitab Ltd, Coventry, UK). Bacterial numbers were log_{10}-transformed before calculations. Statistical differences between means were tested using One-way ANOVA (Tukey multiple comparisons) when more than two means were compared and two-sample t-test for comparison between two mean values. Statistically significant difference was set for differences resulting in p-values below 0.05.

Results

ATP and chemical content of fish associated samples

In general, the Clean-Trace system measured systematically almost 10 times higher RLU values than UltraSnap/Hygiena. For the control without ATP (10 µl dH_2O), the ATP bioluminescence were monitored as 0 and 35-40 RLU for Hygiena and Clean-Trace, respectively (Figure S1). It was a high variation in the content of ATP between the different fractions from trout as measured by both ATP assays. For fractions from raw trout, the highest bioluminescence levels (RLU μg^{-1}) were found in blood and loin, about 100-fold higher than in the belly fat homogenate (Table 1).

Heat treated homogenates showed ATP bioluminescence levels up to >1000 fold higher than corresponding raw fractions. The increase in measured bioluminescence after heat treatment was highest for trout belly fat and beef juice and lowest for trout blood and cod juice (Figure 1). Total bacterial numbers in the samples applied (per 10 µl) in the assay (naturally occurring from raw materials) were <10 cfu in trout fractions, 1800 cfu in cod juice and 3 cfu in beef juice.

220

221 **ATP content of bacteria associated with fish processing**

222 The highest ATP bioluminescence among the bacteria were found for the two *Acinetobacter* sp.
223 (Figure 2). Overall, the ATP levels of cells of the gram positive *Listeria* strains were lower ($p<0.05$)
224 than other bacteria which were all gram negative. There were no significant differences in ATP
225 content between the five *L. monocytogenes* strains. The results for *L. innocua* was similar as for *L.*
226 *monocytogenes*.

227

228 **Influence of processing associated factors on ATP tests**

229 Presence of sodium chloride had an adverse effect on the ATP bioluminescence for both assays
230 tested (Figure 3). For Clean-Trace, sodium chloride reduced the bioluminescence at a concentration
231 as low as 0.9% compared to a control not containing NaCl ($p=0.017$). No significant reduction of ATP
232 bioluminescence was found at 0.9% NaCl for UltraSnap, but it was negatively affected at
233 concentrations ≥4.5%.

234 Liquefied smoke had a negative effect on ATP bioluminescence, measured with both Clean-Trace and
235 UltraSnap (Figure 4), an adverse effect was also seen for 10% liquefied smoke ($p<0.05$). The decrease
236 in presence of liquefied smoke, which had a pH of 2.2, was in the same range as for Glycine HCl
237 buffer with pH 2.2. The ATP reading of 10 µl undiluted liquefied smoke was 0 and 19 RLU with
238 UltraSnap/SystemSURE and Clean-Trace, respectively.

239 The fish oil did not affect the ATP measurement (data not shown). The ATP reading of 10 µl undiluted
240 fish oil was 0 and 63 RLU with UltraSnap/SystemSURE and Clean-Trace, respectively.

241

242 **ATP measurements in fish processing plants**

Trout and salmon processing plant

During a 5 month period, 23.1% of samples from the slaughter department and 1.9% from the filleting department had ATP >100 RLU, which was the critical limit set by the plant. During the same period, the plant also used Hygicult TPC dipslide to measure bacteria at surfaces, and 1.9% of the samples from slaughter- and 0.8% from the filleting department had unacceptable high bacterial numbers (>80 cfu cm^{-2}).

White fish processing plants

Only sites that were visible unclean (with a bacterial level of 5-7 log cfu cm^{-2}) were chosen for microbial monitoring and ATP monitoring using UltraSnap. The average ATP bioluminescence of sites with visible salt residues (5 samples, average 5.7 log cfu cm^{-2}) was 182 RLU whereas sites without visible salt (13 samples, average 6.2 log cfu cm^{-2}) was 43 400 RLU.

Discussion

Hygienic control using ATP-monitoring instruments are widely used by the food industry as a rapid and cost effective way to discover failure in the sanitation processes. Surprisingly, although marketed as a monitoring method for food debris, scientific documentation on the level of ATP, as measured by the ATP-bioluminescence method, in different food soils is scarce. Also, how processing conditions and components affect the results is practically unknown, with the exception of the role of disinfecting agents. The variation in ATP bioluminescence between different types of raw fish fractions was considerable, with the highest ATP values observed for blood. Heat treatment increased the ATP bioluminescence substantially for trout belly fat and loin, as well as beef juice. To our knowledge such an effect of heat treatment on ATP measurement has not been reported previously, for any types of food soil. As heat treatment in itself unlikely result in a higher ATP-

content, it seemed to be a substantial underestimation of ATP content in raw trout loin and belly fat. The ATP assay is dependent on lysis of cells to release ATP, and this effect is obtained by a lysis/extracting solution supplied with the ATP sampling swabs. It is reasonable to believe that some types of cells (e.g. fat cells) are more difficult to lyse than other cells (e.g. blood cells), and that the heat treatment of the former increase the lysis or facilitate ATP extraction so more of the ATP will be available for measurement. For the trout soils, the underestimation of ATP was highest in belly fat, which could be indicating that raw fat in itself inhibited the ATP measurement. However, this hypothesis was rejected as we conducted a control experiment showing that pure fish oil did not affect ATP measurement. Thus it is more likely that the underestimation in raw fat-rich soil is due to limited availability of ATP for the measurement system, e.g. caused by insufficient lysis as suggested above.

As the ATP tests are designed to be used to swab surfaces, we performed control experiments to test whether the main conclusions obtained when applying soils to the swabs with a pipette also were supported in experiments where the swabs were used on a surface. Experiments where 50 µl soil was applied to a 2 X 2 cm coupon of stainless steel and dried for 2 h in a safety hood before swabbing the surface with a ATP swab, showed that the bioluminescence of heat treated trout belly fat homogenate was about 100 times (for both UltraSnap and Clean-Trace swabs) higher than for raw belly fat. Also, the bioluminescence of raw blood was about 100 times (for UltraSnap and Clean-Trace) higher than for raw belly fat homogenate. Thus the main conclusions obtained for adding soils directly to swabs were confirmed with soils dried on surfaces before swabbing.

Other studies have also demonstrated differences in ATP content of different types of food and food soils using bioluminescence principles for monitoring. In a previous study where different type of food matrices were tested with the Accupoint ATP measurement system, the highest ATP signals were from orange juice, followed by yogurt, ground beef, deli turkey and flour (Viator et al. 2017). Whitehead et al. (2008) measured ATP content (ATP measurement systems from Hygiena) of

292 different types of food soil, including heat treated fish (cod) extract, which had comparable ATP
293 bioluminescence levels compared to meat and cheese extract. Although both studies found
294 differences in ATP levels between different types of food matrices they did not point out differences
295 between raw and heat treated products. Another difference between these studies and the present
296 study, is that the latter compare different variants of residues that will be found along the same
297 processing line.

298 In this investigation it was shown that two ATP assays tested were adversely influenced by sodium
299 chloride, which is widely used in the fish industry, e.g. for production of smoked salmon/trout and
300 salt-cured cod. Sodium chloride had a quenching effect on the ATP readings even at as low
301 concentrations as 0.9%. The low ATP readings may be due to an adverse effect of sodium chloride on
302 luciferase activity since the enzyme is inhibited in presence of sodium chloride concentrations as low
303 as <1% (Ishida et al. 2003), due to chloride binding to the active site of the enzyme (Ishida et al.
304 2003). If this hypothesis is correct, this quenching effect may also effect other ATP systems than the
305 two tested in this study, as the same luciferase enzyme is used in many systems, however this
306 remains to be tested.

307 Liquid smoke adversely affected the ATP measurements, probably because of its low pH. A similar
308 reduction in luminescence was found by reducing pH to 2.2 as adding liquid smoke (with pH around
309 2.2). The mechanism of action could be inhibition of the luciferase enzyme which has an optimum for
310 light emission at pH 7.8 (Lundin et al. 1976). Based on the adverse effect of sodium chloride on ATP
311 readings, the sodium chloride concentration of liquid smoke measured. The sodium chloride
312 concentration was below the detection limit of the assay (<0.1%) and it was concluded that the
313 adverse effect of liquid smoke observed could be mainly explained by pH.

314 Among the bacteria tested, the highest ATP levels were observed for *Acinetobacter* sp. (Figure 2),
315 with ATP bioluminescence values corresponding to a detection limit of 50-100 cfu. For the other
316 bacterial genera commonly present in fish industry the detection limit with ATP measurement was

around 1 000 cfu. The results are within the lower range of what found in other studies, where the detection limit with ATP devices has been reported to be in the area of 10^2 – 10^8 cfu, dependent of both the growth phase and the type of bacteria (Turner et al. 2010; Vogel et al. 2014). As reported above, the ATP levels of several types of food soils were high, with detection limits in the low µg range. The ATP content in eukaryotic cells of food residues are reported to be much higher than in bacterial cells (Dostalek and Branyik 2005; Turner et al. 2010). The suggested minor role of bacteria as the source of ATP in hygiene monitoring as can be deduced from the present study is to some extent supported by investigations from food processing surfaces. Some studies from the food production show a correlation between ATP bioluminescence and bacterial counts on surfaces (Osimani et al. 2014; Hammons et al. 2015), while a poor correlation is observed in other studies (Poulis et al. 1993; Moore and Griffith 2002). It was observed that the bacteria usually dominating in fish processing plants had higher ATP values than the pathogenic bacterium *L. monocytogenes*. The RLU per million cfu was more than 1000 times lower for *L. monocytogenes* than for *Acinetobacter* sp. Since the ATP signal of *L. monocytogenes* is low, and *L. monocytogenes* is also usually present in lower numbers than other bacteria in the industry (Møretrø and Langsrud 2017). Taken together this shows that it is difficult to use ATP as a direct measure of bacterial levels after cleaning and disinfection, especially for subgroups like *L. monocytogenes* representing a minority of the total number of bacteria. To our knowledge, only one study have reported on correlation between ATP readings and *L. monocytogenes* prevalence (Hammons et al. 2015). However as bacterial contamination on surfaces are believed to be linked to soiling of surfaces and inadequate sanitation, the ATP readings from food residues may be correlated to the risk of bacterial contamination.

The main findings in this study was supported by measurements with both ATP luminometers tested. Also, several of the conclusions are likely to be relevant also for other types of ATP measurement systems since they all use the luciferase enzyme. However, it cannot be ruled out that differences in the composition of the lysis solution between ATP swabs can lead to different results for different types of food.

According to the manufacturers of the ATP instruments, each user should validate the method against their own standards to set critical limits for corrective actions, but it is common to use "rules of thumb" based on general criteria for hygiene. Some users get sceptical to the method because their expectations of a correlation with bacterial counts are not fulfilled, although such a correlation is not claimed by manufacturers nor supported by the scientific literature on the topic. The results from the laboratory tests in the present study, such as the large variation in ATP-content measured for different fractions of trout and bacteria commonly found after sanitation as well as the huge impact of processing factors, such as heating, salting and smoking, raises a lot of questions about how to interpret ATP-monitoring data from the food industry. One single processing line in the fish production plant may include zones with mainly debris containing blood (slaughtering), fat (gutting), protein rich muscle (filleting) and cooked, salted or smoked residues. The laboratory findings indicated that using the same critical limits for all parts of the process could potentially lead to corrective actions where the hygiene is sufficient (e.g. slaughtering where traces of blood would give a very high ATP-signal) and lack of corrective actions where corrective actions should be taken (e.g. "high hygiene zone" such as the smoking department where traces of salt and low pH could result in low ATP signals even for spots with high numbers of *L. monocytogenes* and soil). To investigate this further in real conditions, historical data from a trout factory (covering slaughter to fillet) were analysed and two factories producing salt-cured cod (covering areas with high and low salt content) were visited and sampled.

The results from the salt-curing cod factories suggested that the presence of salt may indeed result in false negatives in hygiene monitoring since low ATP readings were obtained from sampled surfaces with bacterial counts as high as 10^5 cfu cm^{-2} and visible soil. In salting areas, large amount of salt is used and salt are often visually present on equipment/machines during production. Although the sanitation process will remove the majority of the salt, the ATP test is so sensitive to salt that it is likely the ATP readings in salting areas may occasionally be underreported due to presence of sodium chloride.

The results from analyses of historical data supported our hypothesis that using the same critical limits for different parts of the processing line can result in either under- or overestimation of the hygiene for some parts of the line. Overall, the ATP levels were much higher in the slaughter- compared to the filleting department, while the bacterial numbers were quite similar for both departments. The low correlation of ATP vs bacterial numbers between the departments indicates that careful interpretation of ATP results is important. The same critical limit was used for the whole processing plant and the ATP results indicated that the hygiene in the slaughterhouse was considerable worse than in the filleting department, despite the fact that bacterial results taken over a long period of time showed very small differences between the departments. The explanation of this is unknown but may be based on different types of soil present in the two departments. In the slaughterhouse there is likely to be more blood present which can give high ATP readings, while in the filleting department there is less blood and more of other types of soil e.g. muscle and fat, resulting in lower ATP bioluminescence.

In conclusion, the output from ATP bioluminescence instruments depends not only on the hygienic level after sanitation, but on the nature of food residues present. ATP bioluminescence instruments are widely used by the fish industry for monitoring hygiene today and our results shows that ATP measurements can be used to detect most types of fish fractions that could potentially be present after cleaning and disinfection. However, ATP meters will only give valuable information about the hygiene if critical limits are set after a validation period, distinguishing between areas with different type of soil and the hygiene zone. ATP monitoring should also only be used as a supplement to, and not as a replacement of regular microbiological sampling and a specific seek-destroy surveillance system for *Listeria monocytogenes*.

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Conflict of interest

No conflict of interest declared

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Table 1. ATP (as measured by ATP assays) and chemical content of trout fractions

Samples*	RLU UltraSnap†	RLU Clean-Trace†	Fat (%)	Protein (%)	Dry matter (%)	NaCl (%)	pH
Loin	3360 (301)	16656 (1659)	11.0	22.8	35.2	0.13	6.2
Belly fat	24 (2)	323 (41)	70.9	6.6	87.9	0.15	6.4
Blood	4590 (537)	30131 (5668)	1.8	5.1	11.4	1.18	7.1

*All samples are based on raw fish

†All ATP measurements are for 10 µg sample applied to swabs, and are mean (standard deviation) of 4-6 measurements on two individual tubes of fish fractions. All RLU values are corrected for dilutions performed before measurements

Figure legends

Figure 1. Effect of heat treatment (80 °C, 30 min) of fish and beef soils on ATP bioluminescence. Fold increase in ATP bioluminescence by heat treatment (means and standard error of a total of four heat treated samples) shown for each food fraction and two instruments.

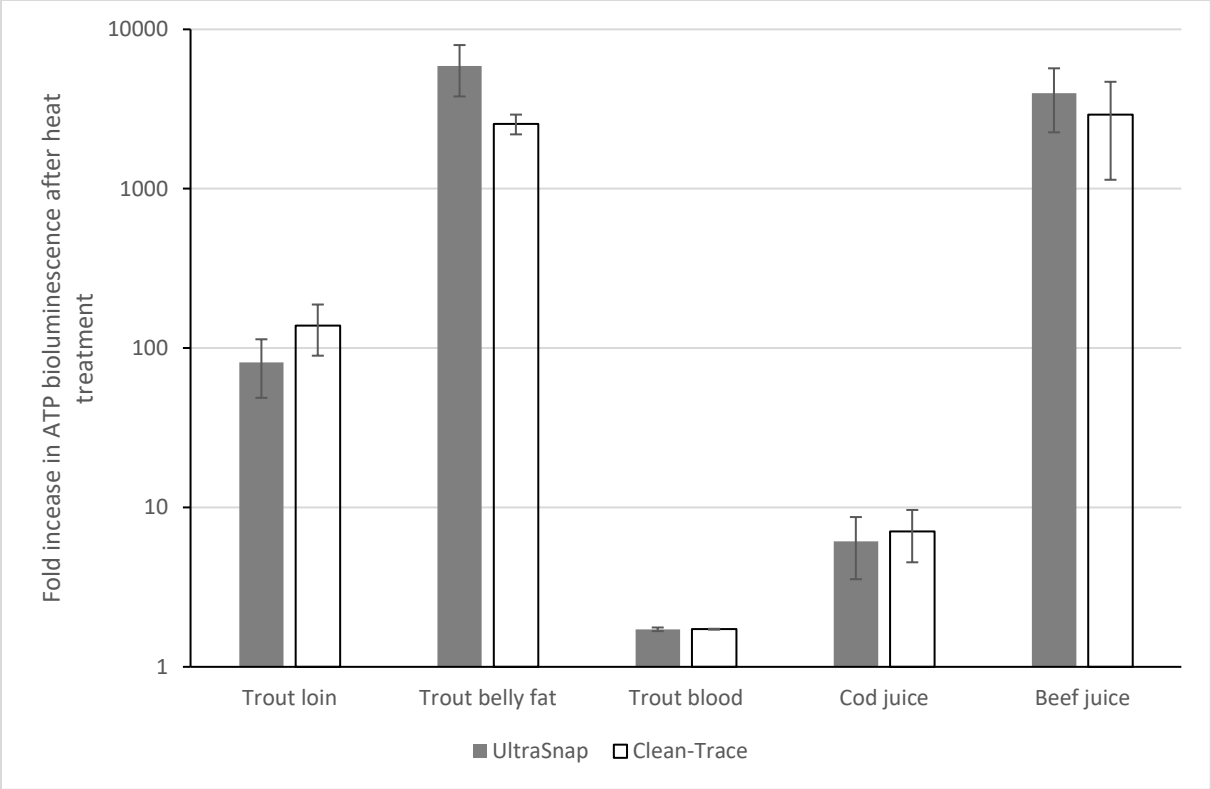
Figure 2. ATP Bioluminescence of bacterial strains. ATP bioluminescence for 1 million bacterial cells (RLU 10^{-6} cfu) (mean values and standard error of three replicates) shown for 15 bacterial strains belonging to 7 genera and using two instruments.

Figure 3. Effect of sodium chloride on ATP bioluminescence. Reduction in bioluminescence (%, means of three replicates and standard errors) plotted against sodium chloride concentration shown.

Figure 4. Effect of liquid smoke on ATP bioluminescence. Reduction in bioluminescence in the presence of 50% liquid smoke (pH 2.2), 5% liquid smoke or in 200 mM Glycine HCl (pH 2.2) (% reduction, means of 2-3 replicates and standard errors shown for each condition).

Legend Supporting information Figure:

Figure S1. Relative units measured as a function of ATP content (femtomol, 10^{-15} mol) added to swabs.



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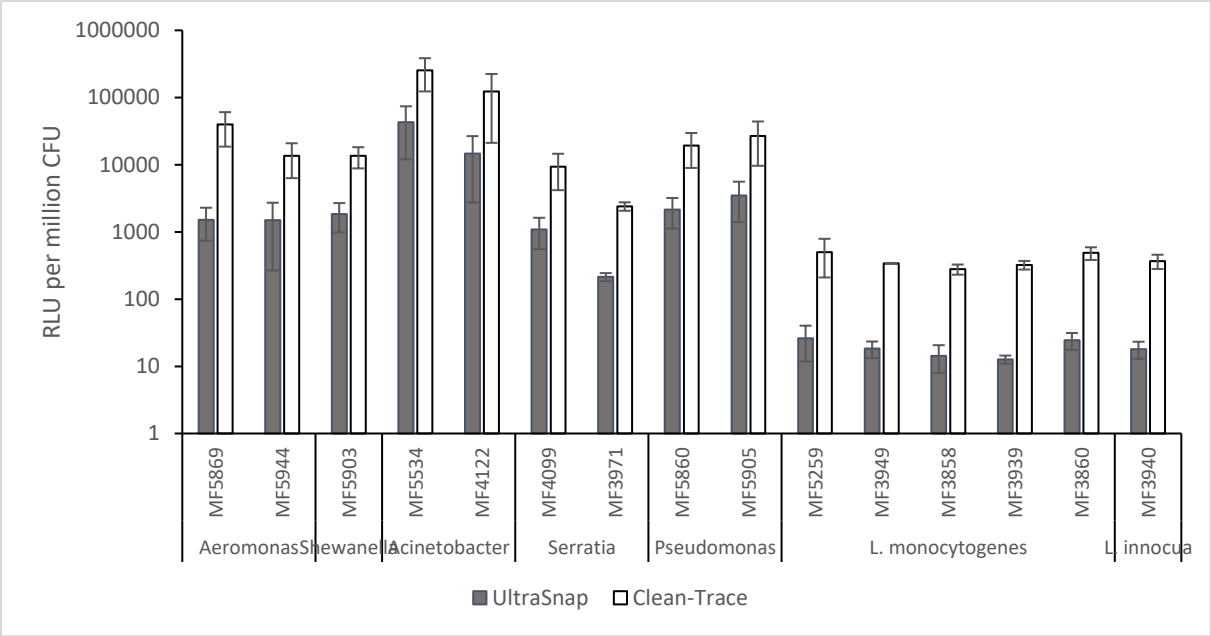

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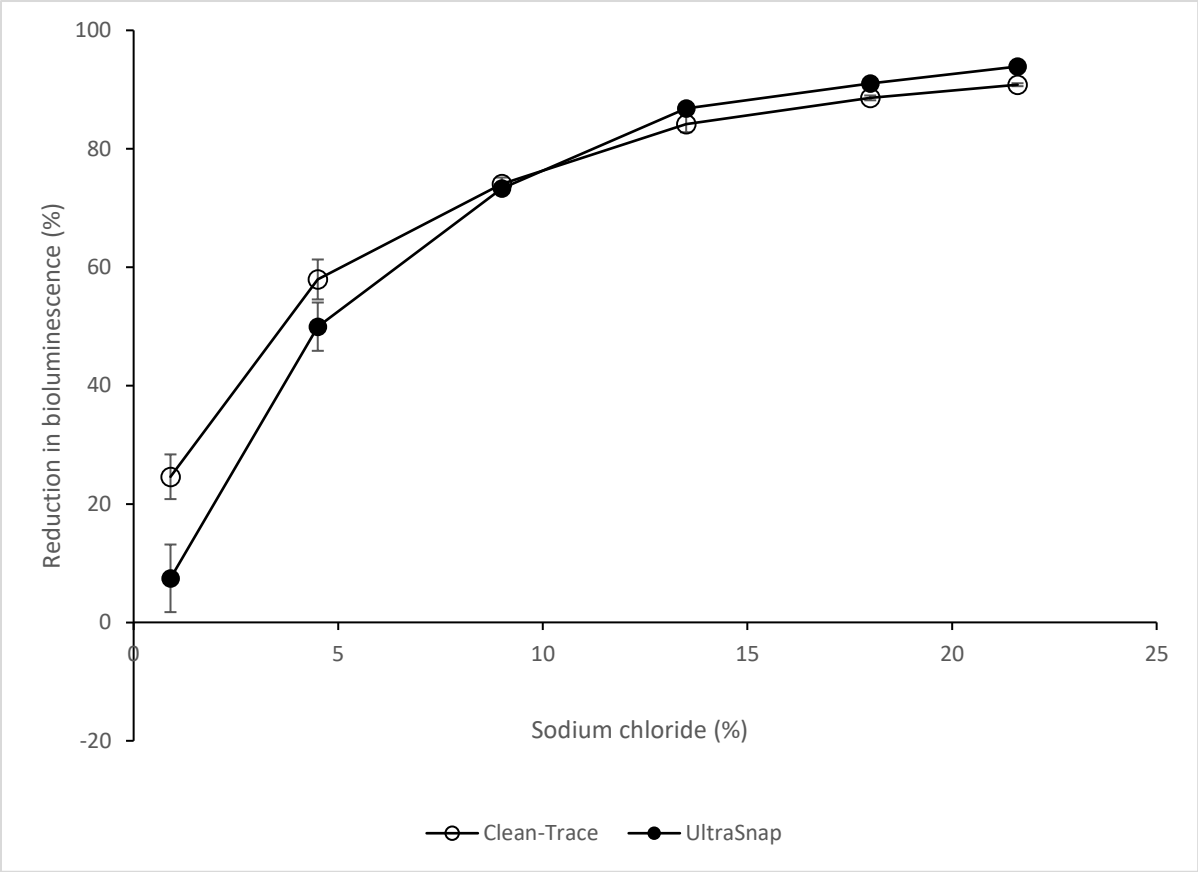

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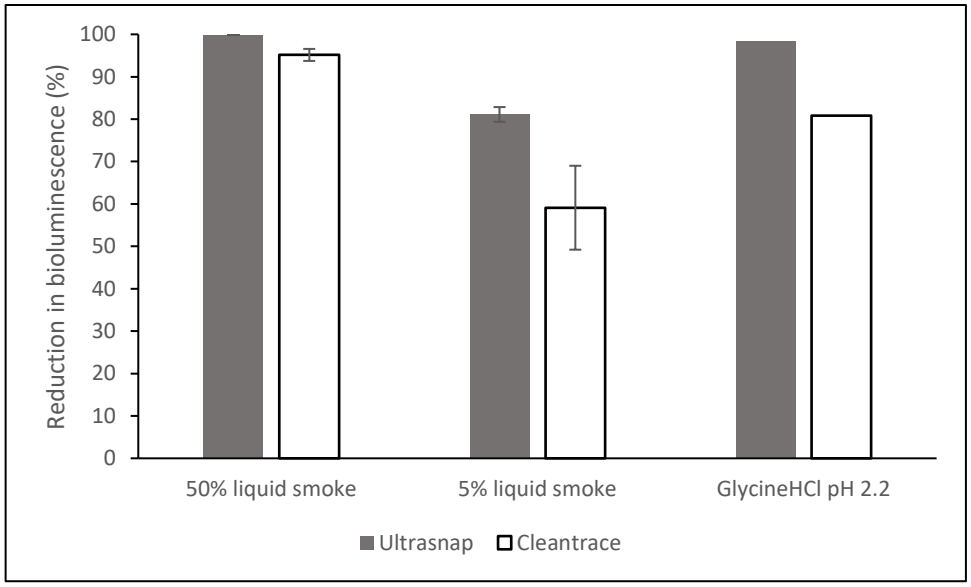

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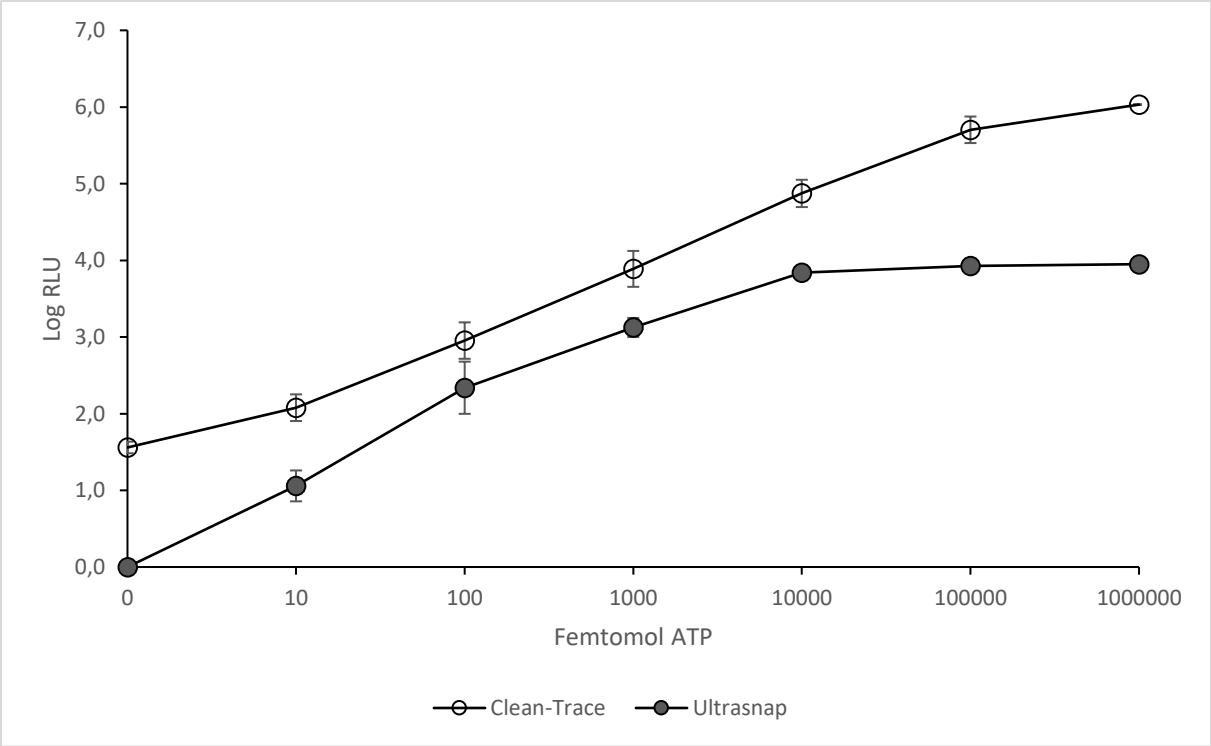
Supporting information

Linearity and sensitivity of ATP reading systems

Initially, the linearity and sensitivity of the ATP reading systems were tested by applying 10 µl of tenfold dilutions of pure ATP (Promega Biotech AB, WI, USA) in dH_2O in the concentration range directly to the ATP swabs. The amount ATP added was in the range 1 $x10^{-9}$ to 1X 10^{-14} mol. The test was performed in triplicate with newly made dilution series for each replicate. In the rest of the studies where samples were measured after application with a pipette directly to swabs, samples were diluted if necessary with dH_2O to <3000 RLU for UltraSnap and <100 000 RLU for Clean-Trace, numbers presented are corrected for dilutions

RLU readings with 3M Clean-Trace NG Luminometer with Clean-Trace swabs correlated linearly with ATP (R^2=0.9994) up to 10^{-10} mol ATP. Hygiena SystemSURE Plus measurements with UltraSnap correlated linearly with ATP up to 10^{-12} mol ATP (R^2=0.9791) (Figure S1). At ATP amounts around 10^{-12} mol ATP the Hygiena SystemSURE Plus reach its maximum readout of 9999 RLU. Clean-Trace showed systematically about ten times higher RLU than UltraSnap in the linear range (10-10 000 femtomol), approximately log 1 and 1 RLU per 10^{-15} mol ATP, respectively, in the linear ATP range. (Figure S1). For Clean-Trace the control sample without ATP showed an output of 30-40 RLU, significantly different from zero ($p < 0.05$), while reading for dH_2O with the UltraSnap test was zero.

In further tests in this study, samples to be measured were diluted to an RLU reading that was within the linear area of the ATP test to be used.



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