Concentration and detection of *Salmonella*

**Concentration and Detection of *Salmonella* in Mung Bean Sprout Spent Irrigation Water using Tangential Flow Filtration Coupled with an Amperometric Flow-Through ELISA**

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Running title: Concentration and detection of *Salmonella*

Keywords: *Salmonella*, mung bean sprouts, tangential flow filtration, amperometric immune-sensor, spent irrigation water

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\textbf{ABSTRACT}

The following reports on the development of a culture-free method for \textit{Salmonella} screening of spent irrigation water derived from sprouting mung bean beds. The system used Tangential Flow Filtration (TFF) to non-specifically concentrate cells from large (2 – 10 l) sample volumes. The retentate (100 ml) from the TFF was then flowed over an anti-\textit{Salmonella} antibody modified cellulose acetate membrane. The captured \textit{Salmonella} was detected by reacting with a secondary anti-\textit{Salmonella} and goat anti-rabbit biotin labelled antibody, followed by avidin-tagged glucose oxidase. The hydrogen peroxide generated from the enzymic oxidation of glucose was amperometrically detected at an underlying platinum electrode. It was found that 10 l of 2 log CFU/ml \textit{Salmonella} suspensions could be concentrated to 4 log CFU/ml with 60\% recovery regardless of the flow rate (112 – 511 ml/min) or transmembrane pressure (0-20 Ib in$^2$) applied. The solids content of spent irrigation water negatively affected the filtration rate of TFF. This was most evident in spent irrigation water collected in the initial 24 h of the sprouting period where the solids content was high (4170 mg/l) compared to samples collected at 96 h (560 mg/l). Trials were performed using mung bean beds inoculated with different \textit{Salmonella} levels (1.3 – 3.3 log CFU/g). By using the optimized TFF and flow-through immunoassay it was possible to detect \textit{Salmonella} in spent irrigation water at levels of 2.43 log CFU/ml within 4 h. The integrated concentration and detection system will provide a useful tool for sprout producers to perform in-house pathogen screening of spent irrigation water.
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Sprouted seeds have been implicated in numerous outbreaks of foodborne illness (1). *Salmonella* remains the most common human pathogen associated with sprouts although outbreaks linked to *Escherichia coli* O157:H7 have also been reported (27, 34, 43). To address the increasing number of foodborne illness outbreaks, the U.S. Food and Drug Administration issued guidelines in 1999 to enhance food safety standards within the sprout industry (1). Amongst other measures, the guidelines recommend testing the spent irrigation water, 48 h into the sprouting process, for presence of pathogens with specific reference to *Salmonella* (1). Screening spent irrigation water is preferred over testing sprouts directly due to uniformity, ease of collection and simple analysis (1). More significantly, because irrigation water runs over the sprouts, it is considered to provide a better assessment on the microbiological status of the sprouting seed bed compared to when individual sprout samples are screened (1).

The protocols for sampling spent irrigation water were validated through studies using inoculated and non-inoculated alfalfa (11, 33). The results from the studies indicated that the growth of *Salmonella* on sprouting alfalfa occurred early in the sprouting period and was homogenously distributed throughout the sprout bed (11, 33). However, a study performed by Hora et al (15) illustrated that contamination within sprouting mung bean beds was heterogeneously distributed and the spread of pathogens (*Salmonella* and *Escherichia coli* O157:H7) delayed when introduced at low levels (1 g inoculated beans per 500 g) (16). Consequently, screening spent irrigation water from a single point early in the sprouting period would not provide a reliable measure of the microbiological safety of mung bean batches (15). A more effective screening method would be to screen large volumes of spent irrigation water late in the sprouting period.
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However, large sample volumes are incompatible with standard microbiological techniques which typically involve pre-enrichment followed by selective plating. In addition, because of the time required to perform pathogen screening, spent irrigation samples need to be collected within the initial 48 h into sprouting to ensure that the results are known prior to product release (1). To address such time constraints, there has been a sustained interest in rapid detection methods for screening spent irrigation water. To date the majority of studies have focused on pathogen detection methods based on immuno (latex test) or molecular methods (real-time PCR) (22, 27, 32). Although such techniques are both rapid and sensitive, the sample size applied to the sensor is restricted to μl volumes thereby requiring a pre-enrichment step to increase pathogen levels to the limit of detection. In addition, culture based techniques are unsuitable for on-site testing due to the need for laboratory facilities and incurred time delay (22).

A non-culture based method has been described for screening spent irrigation water derived from alfalfa beds. The method described was based on centrifugation of spent irrigation water (400 ml) to concentrate cells followed by selective detection using PCR (20). The researchers reported 99% recovery of *Salmonella* or *E. coli* O157:H7 with a lower detection limit of 2 log CFU/ml. Although sensitive, it can be considered that centrifugation and molecular based techniques are too expensive for sprout producers to adopt.

In the following an integrated system has been developed based on Tangential Flow Filtration (TFF) in combination with a flow-through electrochemical ELISA to enable sensitive, culture-free, approach to screening spent irrigation water derived from mung bean sprout production (Figure 1). TFF is a cross-flow system that enables high filtration rates of large volumes of sample without excessive pore blocking as experienced with dead-end filtration techniques (26).
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TFF has been used extensively in the biotechnology industry to recover proteins or metabolic products from fermentations (38) with less attention being placed on recovering microbial biomass (13, 26). However, Fu (12) developed a TFF sampling system for testing spent irrigation water derived from alfalfa sprout production. Here, the spent irrigation water sample collected 48 h into the sprouting period was circulated within a TFF system achieving 100-fold concentration of inoculated *Salmonella* or *E. coli* O157:H7 within 2 h. (12).

In the current study, the selective detection of *Salmonella* was achieved using a flow-through electrochemical ELISA-sensor. Flow-through immune-sensors have the distinct advantage over conventional ELISA in terms of greater sample volumes that can be analyzed thereby enhancing sensitivity (4, 36, 44, 45). Here, the sample is allowed to flow over a layer of antibodies to capture the target analyte and subsequently reacted with an enzyme conjugate. The enzymatic product of the conjugate can then be detected either calorimetrically or electrochemically. Electrochemical transduction has the advantage over optical based methods with respect to low cost hardware, robustness and relatively simple design, in addition to multi-analyte sensing using microarrays (25). The two basic formats available for electrochemical based sensors are chromatographic (immune-columns) or antibody modified electrodes. In the chromatographic format, the cells are captured within a column, reacted with an enzyme conjugate along with substrate. The electroactive product is then eluted and detected at a down-stream electrode (25). The alternative approach is to directly or indirectly immobilize antibodies onto the electrode surface thereby enabling low amounts of enzyme conjugate to be used in addition to enhanced signals due to the lower dilution of enzyme product (25). However, a potential limitation of this approach is passivation of the electrode by deposition of proteins and other organic constituents.
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from the sample matrix (6, 28, 35). Therefore, a protective membrane is typically overlaid onto
the electrode to prevent fouling of the electrode whilst at the same time allowing diffusion of the
enzyme product (21, 30). This is the main reason for selecting a low molecular weight enzymatic
product, such as hydrogen peroxide, that readily diffuses across membrane films to the
underlying electrode (41).

The objective of the following study was to develop a robust system for spent irrigation water
screening that could be readily adopted by sprout producers. To mimic the worse-case-scenario,
the integrated system was verified using spent irrigation water derived from sprouting mung
bean beds inoculated with low levels of *Salmonella* (1.3 log CFU/g) which may be encountered
naturally in contaminated seeds (23).

MATERIALS AND METHODS

**Preparation of *Salmonella* suspensions**

The *Salmonella enterica* serovars selected to study have either been implicated in sprout related
foodborne illness outbreaks or are of clinical significance. The *Salmonella* serovars used
(obtained from the culture collection of the Canadian Research Institute for Food Safety, Guelph,
Canada) in the study were Montevideo P2 (kanamycin resistant (42)), Heidelberg, Senftenberg,
Typhimurium DT104, Newport, Meleagridis and Oranienburg. The *Salmonella* were cultivated
aerobically in Luria-Bertani (LB) broth (Oxoid, Basingstoke, UK) at 37°C for 24 h. The cells
were harvested by centrifugation (5000 x g for 10 min at 4°C) and washed once in sterile saline.
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The pellet was finally resuspended in saline to give a final cell density of 7.0 log CFU/ml (OD$_{600}$ = 0.2).

**Preparation of spent mung bean irrigation water**

Batches (500 g) of mung beans (donated by a local sprout producer) were placed into a 10 l container with a perforated base to allow drainage of irrigation water. The container was placed within a secondary vessel to collect the spent irrigation water. The mung beans were soaked overnight in 2 l of water at 25°C. The water was removed and sprouting continued for a further 96 h with daily irrigation using 2 l volumes of sterile distilled water. On each occasion the water was collected and transferred to a sterile bottle and maintained at 4°C until required. Solids content of spent irrigation water was determined using a conductivity meter (Thermo-Fisher, Whitby, Ontario, Canada).

**Enumeration of *Salmonella* in spent irrigation water derived from sprouting mung bean beds**

Suspensions (500 ml) of *Salmonella* Montevideo P2 were prepared as described above. Mung beans (100 g) were steep inoculated in the bacterial suspension (7 log CFU/ml) for 20 min and subsequently transferred to blotting paper and allowed to dry overnight at room temperature (*ca.* 23°C). Initial loading of the mung beans was determined by placing 1 g of inoculated beans into 9 ml 0.1% peptone and vortexed for 60 s to release the attached cells. Serial dilutions were prepared in 0.1% peptone and plated onto the LB$_{KAN}$ agar (LB containing 30 µg/ml kanamycin).
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Different weights (1, 10 or 100 g) of inoculated beans were introduced into non-inoculated mung bean batches to give a total weight of 500 g. The mung beans were soaked in 2 l water for 24 h at 25°C to stimulate germination. The beans were transferred to 10 l containers as described above and sprouting continued for up to 96 h with daily irrigation using 2 l volumes of water. *Salmonella* levels within the spent irrigation water samples were determined by preparing a dilution series and plating onto LB\textsubscript{KAN} that was subsequently incubated at 37°C for 24 h. When no counts were recovered on plates, 100 ml volumes of spent irrigation water were passed through a sterile filter (47 mm, 0.45\textmu m pore size; Thermo-Fisher, Whitby, ON, Canada) and overlaid onto an LB\textsubscript{KAN} agar prior to incubating at 37°C for 24 h.

Presence/absence tests for *Salmonella* were performed by adding 200 ml sample with 25 ml 0.1% peptone water and incubating at 37°C for 24 h. Aliquots (0.1 ml) was spotted onto semi-solid RV agar (Oxoid) and incubated at 42°C for 20 h. Presumptive positive samples were plated onto XLD agar and incubated for 24 h at 37°C. The Oxoid *Salmonella* Latex Test FT0203 was used for serological confirmation of typical colonies (i.e. red colonies surrounded by brilliant red zones).

**Tangential flow filtration**

The Tangential Flow Filtration (TFF) system, developed by Fu et al. (12), was adopted. The systems uses a MiniKros module composed of hollow fiber polysulfone membranes with a 0.72 m\textsuperscript{2} working area and 0.2 \textmu m pore size (Spectrum Labs, CA, USA). Other components of the TFF system were a 10 l capacity holding chamber, Polycap 36HD pre-filter (10 \textmu m pore size, Spectrum Labs), peristaltic pump (Masterflex Economy Pump, Cole-Parmer, Vernon Hills, IL,
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USA), pressure gauges at the in- and out-let of the TFF filter (Figure 1). The sample to be concentrated was placed in the 10 l holding chamber and re-circulated in a closed loop system through the TFF filtration unit. The flow rate was adjusted by the speed of the pump with the inlet and outlet pressure being controlled by tightening screw clamps (Thermo-Fisher) on the retentate side of the TFF.

The transmembrane pressure and flux rate were calculated using the following equations:-

\[
\text{Transmembrane Pressure} = \frac{\text{(feed pressure} + \text{retentate pressure})}{2} - \text{filtrate pressure}
\]

\[
\text{Flux Rate} = \frac{\text{filtrate rate (h)}}{\text{area of the membrane (m)}}
\]

The filtrate was collected in a secondary container and filtration continued until the dead-volume of the system (100 ml) had been attained. The percent capture was calculated using the equation

\[
\% \text{ Capture} = \frac{\text{Salmonella CFU in Retentate}}{\text{Salmonella CFU original suspension}} \times 100
\]

**Clean-In-Place of TFF filter cartridge**

Three sanitizers were evaluated for Clean-In-Place regimes for regenerating the TFF system. The sanitizers tested were sodium hypochlorite (200 mg/l); acidified sodium chlorite (200 mg/l); and sodium hydroxide (200 mg/l). For each test the filter was used to concentrate a 2 l *Salmonella* cell suspension (5 log CFU/ml). Upon completion of the concentration run the TFF was back-flushed using 5 l sterile distilled water followed by a 20 min circulation of 1 l volumes of the selected sanitizer. Residual sanitizer was then removed by back-flushing with a further 5 l of sterile distilled water. To verify the efficacy of sanitation, a 2 l volume of saline was 9
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circulated through the TFF until the 100 ml dead-volume had been attained. The feed, retentate, permeate, final back flush, and second retentate were screened for the presence of *Salmonella* by plating onto LB$_{KAN}$ agar as previously described.

**Construction of flow through immuno-assay**

The electrochemical cell consisted of a 3 mm diameter platinum electrode with a Ag/AgCl outer electrode that served as the counter and reference (Rank Brothers, Cambridge, UK). The electrode was linked to a Solartron 1260 potentiostat (London Scientific, London Ontario, Canada) and polarised at 0.65V vs Ag/AgCl. The current response was recorded and data processed using CorrWare (Scribner, Southern Pines, North Carolina, USA). The retentate was placed in a holding chamber as fed into the electrochemical cell via a peristaltic pump (Thermo-Fisher) operating at 0.89 ml/min. The volume above the membrane was set at 0.5 cm via a plunger which was placed into the electrochemical cell.

The working electrode was overlaid with a membrane that served as a surface to immobilize the capture antibody and semi-permeable interface to permit diffusion of hydrogen peroxide (product of glucose oxidase enzyme conjugate), as well as a means of minimizing electrode fouling. The membranes evaluated for this purpose were plasticized polyvinyl chloride (PVC), cellulose acetate and poly phenyl ether sulphone (PES). PVC was prepared by dissolving 60 mg of PVC (MW 43000, Sigma-Aldrich, Oakville, ON, Canada) in 10 ml tetrahydrofuran (THF) containing 100 µl Tween 20. The solution was cast into a glass petri-dish (100 mm diameter) and solvent evaporated over-night at room temperature (*ca.* 23°C) within a fume-hood.
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Poly(phenyl ether sulphone) (60 mg, Sigma-Aldrich, Oakville, ON, Canada) was dissolved in 10 ml chloroform and cast into a glass petri-dish (100 mm diameter). Cellulose acetate (0.1 g) (MW 30000, Sigma-Aldrich) was dissolved in 10 ml acetone and cast into a glass Petri dish. In each case the solvent was allowed to evaporate at room temperature within a fume-hood. The permeability of the membranes to hydrogen peroxide was assessed by overlaying the working electrode with 1.5 cm² sections of the test films. An aliquot (2 ml) of KCl (0.5 M) was added to the electrochemical cell that was stirred via a magnetic stirrer. The working electrode was polarized by applying 0.65 V vs Ag/AgCl and the current allowed to attain a steady baseline. A step-calibration curve was constructed by the sequential addition of aliquots (0.1 ml) of hydrogen peroxide (1 mM, Thermo-Fisher). The response was determined by subtracting the steady-state current from the baseline.

To assess the anti-fouling properties of the different membranes, step-calibration curves were performed in the presence of bovine serum albumin (10 mg/ml; Sigma-Aldrich) or a 7.0 log CFU/ml heat killed *Salmonella* suspension.

**Capture antibody immobilization**

A mouse monoclonal anti-*Salmonella* IgG antibody, with specificity to the LPS core of Groups A - E, (Abcam, Cambridge, MA, USA; ab20949) was immobilized onto cellulose acetate membranes using a thiolation reaction. The reactive gold layer was deposited onto cellulose acetate membranes by sputter coating at 15 mV for 2 min. The monoclonal anti-*Salmonella* antibody was diluted to 0.5 mg /ml in PBS. Three cross-linker solutions (all obtained from Sigma-Aldrich) were used to covalently attach a thiol group to the antibody; specifically: 20 mM
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dimethyl-3,3’-dithiobispropionimidate·2HCl (DTBP) (20 mM bicarbonate buffer, pH 9.0); 20 mM 3,3’-dithiobis (sulfosuccinimidylpropionate; DTSSP) (5 mM sodium citrate buffer, pH 5.0); and 20 mM each of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido] hexanoate (sulfo-LC-SPDP) in sterile water. Aliquots (3 µl) of each of the antibody and the three cross-linker solutions were gently mixed and incubated at room temperature for 1 h. To reduce the disulfide bond of the now thiololated antibody, 2 µl of dithiothreitol (DTT) (0.1 M sodium acetate buffer, 0.1 M NaCl, pH 4.5) was added to the mixture and reacted for 30 min. The solution was then spread onto the gold layered cellulose acetate membrane and allowed to dry at room temperature for 1 h. The antibody-coated cellulose acetate membrane was then washed consecutively with phosphate buffered saline and sterile distilled water.

Verification of antibody immobilization was performed by flowing 100 ml *Salmonella* suspension (7 log CFU/ml) over the antibody modified cellulose acetate using flow rates of 0.58, 0.69 or 0.89 ml/min. The membrane was then rinsed with sterile water to remove loosely attached cells. The captured cells were fixed onto the membrane by using 2 % glutaraldehyde and 1 % osmium oxide followed by dehydration in ethanol solutions as described by Gamliel (14). The membrane was then sputter coated with gold and viewed under a Hitachi S-3700N Scanning Electron Microscope. (Hitachi, Pleasanton, CA).

Capture efficiency of the flow-through immune sensor was determined using 100 ml *Salmonella* suspensions (100 ml) diluted from 7 log CFU/ml to 2 log CFU/ml. The suspension was circulated around the flow system at a rate of 0.89 ml/min (total time 2 h). Samples (1 ml) were removed at the start and end of the flow cell process and plated onto LB$_{KAN}$ or XLD prior to
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incubation at 37°C for 24 h. The capture efficiency was calculated by comparing *Salmonella* in the original suspension to levels retained within the suspension following the completion of the circulation through the immune-sensor. The degree of non-specific binding was determined by using gold spluttered cellulose acetate membranes containing no anti-*Salmonella* antibodies.

**Electrochemical detection of *Salmonella***

*Salmonella* captured on the antibody modified cellulose acetate membranes were reacted for 20 min at room temperature with 25 μl of a secondary antibody (rabbit polyclonal with affinity for O and H antigens of *Salmonella*, 1 mg/ml; ab35156). The reaction cell was washed with 5 x 1 ml volumes of saline to remove unbound antibody prior to addition of 100 μl of biotin labelled goat polyclonal anti- rabbit (0.02 mg/ml; Abcam, ab6720). The antibody was allowed to attach for 20 min at room temperature prior to rinsing the cell with saline. Finally, 20 -120 μg glucose oxidase avidin conjugated glucose oxidase (Vector Laboratories, Burlington, Ontario, Canada), was applied to the membrane and allowed to bind to the biotin moiety of the anti-rabbit polyclonal for 40 min. The reaction cell was rinsed with sterile water to remove unattached glucose oxidase and 1mlof electrolyte (0.1M KCl) added. The background current was allowed to attain a steady state value prior to the addition of 1 ml glucose (1 M). The increase in current was recorded and subtracted from the background to give the sensor response.

The concentration of active units of glucose oxidase antibody conjugate was determined using dianisidine-peroxidase assay. The assay consisted of 1.25 ml dianisidine-buffer mixture (0.1 % w/v Sigma-Aldrich), 100 μl 20 Purpurgalin units/ml peroxidase (Sigma-Aldrich) and 1 μl of the conjugated glucose oxidase. The reaction was started by the addition of 150 μl 18 % w/v glucose
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and the increase in $A_{460\text{nm}}$ recorded over 5 minutes using a Shimadzu spectrophotometer (Thermo-Fisher). The units/mg enzyme was calculated as follows:

\[
\text{Units/mg} = \frac{\Delta A_{460\text{nm}}}{\text{min}} / (11.3 \times \text{mg enzyme} / \text{ml reaction mixture})
\]

**Statistical analysis and experimental design**

Except where otherwise stated all tests were repeated three times. Means generated were analyzed by analysis of variance and Tukey test. In all cases, the level of significance was set at $P < 0.05$.

**RESULTS AND DISCUSSION**

**Concentration of *Salmonella* suspensions using Tangential Flow Filtration**

For optimization studies, 10 l of 0.8% w/v saline inoculated with 2 log CFU/ml (6 log CFU/10 l) *Salmonella* was circulated through the TFF operating at different flow rates and transmembrane pressure (TMP). The suspension was concentrated within the TFF system until the dead volume (100 ml) had been attained which took 21 min to 3 h depending on the applied flow rate and pressure.

The average concentration factor achieved using the various flow rate and TMP combinations was 126-fold (60% recovery) which is comparable to values previously reported by other workers for concentrating bacterial suspensions (18, 26). Both flow rate and TMP did not have a significant ($P > 0.05$) effect on the levels of *Salmonella* recovered (Figure 2). This may have been unexpected given that flow rate and TMP collectively defined the shear forces imposed on the cells which, if excessive, results in membrane disruption (26). It has been previously reported
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that high shear forces significantly decrease the recovery of yeasts cells by cross-flow filtration (26). However, in the current study it was evident that *Salmonella* was less sensitive to the generated shear forces thereby retaining viability during the filtration process. In practical terms it is desirable to have high flow rates to facilitate rapid filtration and low TMP to reduce the stresses imposed on the TFF thereby extending the working life-time of the filter (39). Spent irrigation water contains solids derived from bean exudates, in addition to microbial biomass and constituents that could impact on the filtration efficacy of TFF. It was found that the solids content of spent irrigation water collected at different time periods during the sprouting period varied significantly (P < 0.05; Figure 3). Spent irrigation water collected shortly after the beans had been soaked in water for 24 h contained the highest solids content compared to samples collected towards the end of the sprouting period (Figure 3). The changes in solids content can be attributed to the release of exudates from the germinating beans that are maximal at the early stages of sprout development (46). Relevant to the current study, the high solids content of the spent irrigation water negatively affected the flux rate of TFF (Figure 3). The effect of solids content on flux rates can be attributed to the accumulation of organic matter within the pores of the TFF hollow fibre that impedes the flow of water through the membrane (2, 9, 29).

The solids content of spent irrigation water collected from mung bean beds 48-96 h into the sprouting period was not significantly (P > 0.05) different. However, the flux rate observed for irrigation water collected at either 48 or 72 h was significantly (P < 0.05) lower compared to samples withdrawn at 96 h (Figure 3). Such differences may be attributed to the composition of solids content. For example, polysaccharide materials results in agglomeration of particulates which would result in increased biofouling compared to low molecular weight constituents such
Concentration and detection of *Salmonella* as organic acids (8, 19). Regardless of this fact the results indicated that spent irrigation water collected at the end of the sprouting period resulted in less biofouling of the membrane and facilitated high filtration rates.

The reduced flux with increasing solids content could be compensated to a degree by increasing the TMP (Figure 3). By using a higher TMP (3.5 lb in²) an increase in shear force is achieved that minimizes the accumulation of organic matter within the pores of the TFF hollow fibre thereby maintaining high flux rates (8).

For optimal recovery of *Salmonella* it is desirable to collect samples when levels of the enteric pathogen within the sprouting mung bean bed are maximal. Therefore, trials were performed to determine the levels of *Salmonella* associated with spent irrigation water collected at different times during the sprouting period. To mimic the worst-case-scenario, *Salmonella* was introduced at low levels (1.3 log CFU/g) into 500 g mung bean batches. The levels of *Salmonella* remained relatively constant during the initial 48 h into the sprouting period then increased thereafter attaining levels of 2.5 ± 0.4 log CFU/ml at 96 h. The results were in agreement with Hora et al (15) who also reported low *Salmonella* levels in spent irrigation water when the enteric pathogens were introduced at comparable cell densities as used in the current study. The low level of *Salmonella* was unexpected given that other reports have demonstrated rapid growth of the pathogen on sprouting seeds (17, 42). However, it should be noted that in the majority of studies to date seeds were inoculated with a relatively high inoculum level (>4 log CFU/g) and also levels of the enteric pathogen were enumerated on sprouts as opposed to spent irrigation water.
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The low *Salmonella* levels within spent irrigation water observed in the current study can be explained by the strong attachment of the human pathogen on sprouts (7). Consequently, *Salmonella* is more likely to be retained within the sprout bed than washed out during irrigation (15). A further possibility is the suppression of *Salmonella* growth by the antagonistic action of endogenous microflora (10). In the current study it was noted that the Total Aerobic Count (TAC) associated with spent irrigation water progressively increased during the sprouting period (Figure 4). Therefore, it is likely that *Salmonella* introduced at low levels could not effectively compete in the early stages of the sprouting period.

It has been recommended that spent irrigation water should be sampled 48 h into the sprouting period (1). Based on the results obtained in this study, sampling early in the sprouting production would reduce the possibility of detecting *Salmonella* if present. However, by performing spent irrigation water screening at the end of the sprouting period, the levels of *Salmonella* are maximal and consequently more likely to be detected (15).

The recovery of *Salmonella* inoculated into spent irrigation water was evaluated. Here spent irrigation water was collected from sprouting mung bean beds 96 h into the sprout production and inoculated with defined levels of *Salmonella* using the 7 log CFU/ml suspension (Table 1). The inoculated spent irrigation water was then concentrated using the optimized TFF. It was found that the cell density of pathogens could be increased by 10 – 16 fold by using TFF (Table 1). The percent recovery of *Salmonella* in spent irrigation water was lower than that observed in saline (Figure 2). In addition, it was noted that the % recovery of *Salmonella* was significantly (P < 0.05) decreased when 2 log CFU/ml suspensions were processed compared to 4.5 log CFU/ml (Table 1). The lower recovery yields with dilute *Salmonella* suspensions could have
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been due to cells binding to the membrane surface which occurs during the filtration process (26).

**Clean-in-Place of Tangential Flow Filtration Unit**

Although the TTF cartridges used in the current study were only intended for single-use, it can be envisaged that in commercial practice the filter would perform multiple runs. This necessitates developing clean-in-place protocols that can ensure inactivation and removal of residual microbial cells without compromising the integrity of the filter membrane. From the sanitizers tested it was evident that sodium hydroxide proved least effective with *Salmonella* being recovered following the CIP cycle. In contrast, both hypochlorite and acidified sodium chlorite were both effective at sanitizing the TFF unit. However, applying acidified sodium chlorite caused accumulation of white crystals within the filter and hence negatively impacted on performance. Consequently, sodium hypochlorite was used in subsequent studies for sanitizing the TFF between pressure runs. In general the filters could be used to process 8 times before failing (i.e. recovery of *Salmonella* from the permeate). No progressive decrease in filtration performance (i.e. capture yield, flux rate) was observed between TFF runs. However, when membrane failure occurred there was an abrupt loss of TMP which coincided with *Salmonella* being recovered in the filtrate.

**Flow-through amperometric immuno-sensor design and optimization**

A flow-through immuno-assay was designed based on passing the retentate derived from the TFF step over a membrane modified with anti-*Salmonella* antibodies. In addition to acting as a solid support, the membrane also functioned as a perm-selective membrane to prevent biofouling
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of the electrode which was used to detect the hydrogen peroxide enzymatic product of the glucose oxidase antibody conjugate. The three different base membrane types evaluated were cellulose acetate, plasticized PVC and poly phenyl ether sulphone (PES). All three polymers are widely used in biosensor devices due to biocompatibility or perm-selectivity characteristics (3, 37). The PES membrane was too brittle and could not be overlaid over the electrode without breaking and PVC was readily passivated by proteins. However, cellulose acetate membranes were both biocompatible and permeable to hydrogen peroxide. Biocompatibility was evaluated by determining the response to hydrogen peroxide in the presence and absence of BSA or heat killed cells. Although the hydrogen peroxide amperometric response was lower in the presence of BSA or heat killed cells this was insignificantly (P>0.05) different compared to when dose response curves were performed in KCl alone (Figure 5).

**Immobilization of antibodies onto cellulose acetate membranes**

Anti-*Salmonella* antibody was immobilized onto the surface of gold sputtered cellulose acetate membranes via thiol coupling. Verification of antibody immobilization was performed by reacting the modified cellulose acetate membrane with diluted *Salmonella* suspensions (6 log CFU/ml). After incubating at room temperature for 40 min the membrane was rinsed to remove non-attached *Salmonella* prior to viewing under SEM. *Salmonella* cells were observed on antibody modified films although negligible numbers were encountered on non-modified membranes (Figure 6). The results confirm that the antibody modified cellulose acetate resulted in specific binding of *Salmonella* with minimal non-specific binding.
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The anti-*Salmonella* modified cellulose acetate membrane was integrated into a flow cell and capture efficiency determined. Here, different *Salmonella* serovar suspensions (100ml) were flowed (0.89 ml/min) over the antibody layer and changes in levels of the enteric pathogen remaining being used to calculate the capture efficacy (Table 2). The capture efficiency of anti-*Salmonella* modified cellulose acetate membranes ranged from 64 – 95% (Table 2). The similar capture efficiencies with the different serovars confirm that the sensor could be used to capture different *Salmonella* types tested.

The level of *Salmonella* captured is comparable to immune-columns (5) and paramagnetic beads (40) both of which report efficiencies of 95%. In the current study the high capture efficiencies could be attributed of the high affinity of the anti-*Salmonella* antibodies. However, it should be noted that the non-specific binding (i.e. films containing no antibodies) was relatively high (12-36%; Table 2). The non-specific binding was likely caused by the attachment of cells to the tubing and reaction cell. However, the capture of *Salmonella* was consistently higher with antibody modified films indicating the majority of binding was specific and not due to passive attachment.

The captured cells were detected by reacting *Salmonella* with a secondary rabbit anti-*Salmonella* biotin-labelled antibody followed by avidin-tagged glucose oxidase. Glucose enzyme substrate was then added and the hydrogen peroxide product detected amperometrically at the underlying platinum electrode. The assay was optimized in terms of assay time, temperature, concentration of antibody and glucose oxidase. The optimal sensor response was found by reacting 6 µl (2 mg/ml) biotin-labelled antibody with 120 µg glucose oxidase at 23°C for 40 min (results not shown).
Evaluation of the integrated tangential flow filtration and amperometric immunosensor to detect *Salmonella* in spent irrigation water

Spent irrigation water (2 l) was collected from mung bean beds initially inoculated with different levels of *Salmonella* (1.3 – 3.3 log CFU/g; Table 3). The level of *Salmonella* recovered from the spent irrigation water at the end of the 96 h sprouting period was dependent on the initial inoculation introduced onto the mung beans (Table 3). The same finding has been reported for the growth of *Salmonella* introduced at different levels within sprouting alfalfa sprouts (24).

The concentration factor when the spent irrigation water was passed through the TFF was found to be dependent on the *Salmonella* concentration in the sample (Table 3). Low levels of *Salmonella* in the spent irrigation water resulted in the lowest concentration factor which was again possibly due to binding of cells to the filtration unit. It was noted that the concentration factors were higher compared to when *Salmonella* was directly inoculated into spent irrigation water (Table 1). This may have been attributed to the physiological state of the cells which enhanced tolerance to shear effects and/or reduced attachment to the filter membrane.

The response of the amperometric immunosensor correlated ($r^2 = 0.91$) with the *Salmonella* levels within the retentate. However, it is unlikely that the sensor could be used to quantify *Salmonella* present within the sprouting mung bean bed given the variability in recovery yield using TFF and capture efficiency of the antibody modified membranes. The lack of quantitative measurement should not be considered a weakness of the sensor given that any *Salmonella* detected would be considered a hazard. In this respect the lower detection limit is of greater significance and as observed, the sensor could detect *Salmonella* concentrations on the order of 2 log CFU/ml following TFF. The lower detection limit if the flow-through immune-sensor in
Concentration and detection of *Salmonella*

isolation is comparable to other reported flow-through immune-sensors which typically can detect levels on the order of 3-4 log CFU/ml (6, 31).

To test the reliability of the integrated TFF-sensor system 20 trials were performed where mung bean beds were inoculated with 1.3 log CFU/g *Salmonella* and sprouted for 96 h (Figure 7). The spent irrigation water (2 l) was passed through the TFF system and retentate transferred to the flow through immune-sensor. Each TFF filter cartridge was re-used seven times with the CIP hypochlorite decontamination step as previously described. A fresh antibody modified cellulose acetate membrane was used on each occasion.

The sensor response was found to be variable although consistently higher than the baseline (spent irrigation water derived from non-inoculated mung bean beds). *Salmonella* was not detected in spent irrigation water taken from the control (non-inoculated) mung bean batches confirming that no false-positives were generated by the sensor. Inoculated mung beans in Trial 2 gave a low response (Figure 7) which was not significantly different to the baseline value and hence can be regarded as a false-negative given that the retentate sample tested positive for *Salmonella* upon enrichment. Several samples resulted in high sensor responses which may be related to variation in the fabricated antibody modified membrane or levels of *Salmonella* within the retentate. However, regardless of this fact the results demonstrated the consistency of the integrated system to detect *Salmonella* within spent irrigation water. Furthermore, *Salmonella* captured on the membrane could be isolated by enrichment and subsequent plating onto selective media thereby enabling conformational tests to be performed in addition to epidemiology investigations.
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In conclusion, the study has provided proof-of-principle for an integrated device to screen spent irrigation water derived from sprouting mung bean beds. The method is highly sensitive and can detect *Salmonella* in spent irrigation water within 4 h. Due to the rapid analysis time it is possible to take samples at the end of the sprouting process when *Salmonella* numbers are high. The system can be potentially automated and TFF cartridges re-used both thereby making the system a commercially viable alternative to current testing procedures. However, further studies are needed to verify the device performance in capturing and detecting a wider range of *Salmonella* serovars that may be encountered in sprouted seed production.

**ACKNOWLEDGEMENTS**

The authors wish to thank the commercial sprout grower for donating mung beans used in the study and Ontario Ministry of Agriculture and Food (OMAFRA) Food Safety Research and Innovation Program (Project FS050633) for their financial support.

**REFERENCES**


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**Figure 1**: Schematic diagram of the integrated Tangential Flow Filtration (TFF) and amperometric flow-through ELISA. The spent irrigation water sample is recirculated through a TFF unit to concentrate bacterial cells. The retentate is then flowed over capture antibodies immobilized on the surface of a cellulose acetate membrane. The captured *Salmonella* are detected via the addition of secondary antibody glucose oxidase conjugate.

**Figure 2**: Effect of TFF flow rate and transmembrane pressure on the concentration of *Salmonella* from a suspension originally containing 2 log CFU/ml. *Salmonella* suspensions were prepared in saline and passed through TFF unit at different transmembrane pressure and flow rate. The cell density of *Salmonella* within the retentate following filtration was then determined and used to calculate the concentration factor.

**Figure 3**: Effect of solids content of spent mung bean irrigation water on the flux rate through tangential flow filtration unit. Mung bean batches (500g) were sprouted for 96h at 25°C and watered daily with 2 l irrigation water. The spent irrigation water was collected and 100ml samples withdrawn for dry weight analysis (♦). The remaining spent irrigation water was passed through TFF with an operating transmembrane pressure at either 3.5 lb in2 (■) or 0.5 lb in2 (▲) and flow rate of 0.51 l/min.
Figure 4: Total aerobic count and Salmonella within spent irrigation water derived from sprouting of mung bean batches at different time periods during sprouting. Batches (500g) of mung beans were inoculated with Salmonella (1.3 log CFU/g). The beans were sprouted over 96h at 25°C and irrigated daily with 2 l of water. The spent water was collected and Salmonella levels determined.

Figure 5: Response of a cellulose acetate modified platinum electrode to hydrogen peroxide in the presence of 0.1% BSA (A) or heat killed (4 log CFU/ml) Salmonella (B). Step calibration curves were performed by addition of 1mM hydrogen peroxide (♦) to a polarized (0.65V vs Ag/AgCl) platinum electrode modified with a cellulose acetate membrane. The dose response to hydrogen peroxide was repeated in the presence of BSA or heat killed cells (■) using the same cellulose acetate membrane. Finally the dose response of the cellulose acetate membrane to hydrogen peroxide alone was assessed to determine the degree of biofouling (▲). Data points represent the average of three different cellulose acetate membranes.

Figure 6: Scanning electron micrographs of cellulose acetate membranes without (A) and with (B) immobilized capture antibody. A thin gold layer was deposited onto the surface of cellulose acetate membranes upon which capture antibody was immobilized. The membrane was reacted with a Salmonella suspension and subsequently was to remove unbound cells. The control was prepared in the same way except that no capture antibody was immobilized on the membrane surface.
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*Figure 7: Verification of integrated Tangential Flow Filtration and immune-sensor to detect Salmonella in spent irrigation water collected from mung bean beds 96 h into the sprouting process.* Salmonella was inoculated into mung bean batches (1.3 log CFU/g) and sprouted over 96 h with daily irrigation with 2 l water. At the end of the sprouting period, 2 l of spent irrigation water was collected and concentrated using TFF. The retentate was then applied to the amperometric ELISA as described in the legend to Table 4. For sensor response for individual samples was plotted (■) and compared to the average response obtained for samples derived from non-inoculated controls (-----).
Table 1: Increased concentration and percent recovery after concentrating 2 l of spent irrigation water containing Salmonella. Spent irrigation water was collected from mung bean batches sprouted over 96h at 25°C. The spent irrigation water was inoculated with Salmonella and passed through the TFF system. The levels of target bacteria were enumerated from the retentate and used to calculate the concentration factor and percent recovery. TMP 3.5 Ib in², flow rate 511 ml/min

<table>
<thead>
<tr>
<th>Initial (log CFU/ml)</th>
<th>Retentate log CFU/ml (CF(^a))</th>
<th>% Recovery(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>5.7±0.58 (15.85)</td>
<td>82 ± 8</td>
</tr>
<tr>
<td>2.0</td>
<td>3.0±0.13(10.00)</td>
<td>55 ± 8</td>
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</table>

\(^a\)CF: Concentration factor = CFU retentate/CFU initial suspension

\(^b\) % Recovery = (total CFU retentate/total CFU initial suspension)*100
Table 2. *Capture of different* Salmonella *serovars on anti-Salmonella modified and non-modified cellulose acetate membranes.* Salmonella suspensions (3 log CFU/ml) were flowed over (0.89 ml/min) cellulose acetate membrane and levels of the enteric pathogen retained within the suspension enumerated.

<table>
<thead>
<tr>
<th>Salmonella serovar</th>
<th>Log CFU/ml anti-Salmonella CA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CA&lt;sup&gt;a&lt;/sup&gt; control (% Capture)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Log CFU/ml CA&lt;sup&gt;a&lt;/sup&gt; control (% Capture)&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Heidelberg</td>
<td>2.17 ± 0.35 (90%)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.96 ± 0.17 (36%)&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>Senftenberg</td>
<td>1.88 ± 0.39 (95%)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.10 ± 0.31 (12%)&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>Montevideo</td>
<td>2.58 ± 0.23 (73%)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.03 ± 0.32 (25%)&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>Newport</td>
<td>2.12 ± 0.20 (91%)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.06 ± 0.29 (20%)&lt;sup&gt;B&lt;/sup&gt;</td>
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<td>Meleagridis</td>
<td>2.71 ± 0.21 (64%)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.04 ± 0.31 (24%)&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Oranienburg</td>
<td>2.39 ± 0.32 (83%)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.99 ± 0.32 (32%)&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>CA: Cellulose acetate

<sup>b</sup>% Recovery = (CFU Remaining in suspension/total CFU Initial)*100

Values within rows followed by the same letter are not significantly different
Table 3: Concentration and detection of Salmonella from spent irrigation water derived from sprouting mung bean beds. Salmonella was inoculated into mung bean batches (1.3 -3.3 log CFU/g) and sprouted over 96 h. Spent irrigation samples (2 l) were collected at the end of the sprouting period and circulated through tangential flow filtration unit (511 ml/min, TMP 3.5 lb in²). The retentate from the TFF was then passed through immune-flow cell (0.98 ml/min) and captured cells reacted with secondary glucose oxidase conjugate. The amperometric sensor response upon addition of glucose substrate was then recorded.

<table>
<thead>
<tr>
<th>Initial Loading log CFU/g</th>
<th>Spent Irrigation Water log CFU/ml</th>
<th>Retentate log CFU/ml (CF)</th>
<th>Sensor Response (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>32.6±19.82^A</td>
</tr>
<tr>
<td>1.3</td>
<td>2.43±0.39</td>
<td>3.0±0.13 (4)</td>
<td>342.1±135.04^B</td>
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<tr>
<td>1.9</td>
<td>3.74±0.36</td>
<td>5.7±0.58 (91)</td>
<td>364.9±137.10^B</td>
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<tr>
<td>3.3</td>
<td>4.17±0.46</td>
<td>7.47±0.46 (3715)</td>
<td>668.4±71.91^C</td>
</tr>
</tbody>
</table>

^A^CF: Concentration factor = CFU retentate/CFU initial suspension

Means followed by the same letter are not significantly (P<0.05) different
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