# The influence of the microbial quality of wastewater, lettuce cultivars and enumeration technique when estimating the microbial contamination of wastewater-irrigated lettuce

P. Makkaew, M. Miller, N. J. Cromar and H. J. Fallowfield

## ABSTRACT

This study investigated the volume of wastewater retained on the surface of three different varieties of lettuce, Iceberg, Cos, and Oak leaf, following submersion in wastewater of different microbial qualities (10, $10^2$, $10^3$, and $10^4$ *E. coli* MPN/100 mL) as a surrogate method for estimation of contamination of spray-irrigated lettuce. Uniquely, *Escherichia coli* was enumerated, after submersion, on both the outer and inner leaves and in a composite sample of lettuce. *E. coli* were enumerated using two techniques. Firstly, from samples of leaves – the direct method. Secondly, using an indirect method, where the *E. coli* concentrations were estimated from the volume of wastewater retained by the lettuce and the *E. coli* concentration of the wastewater. The results showed that different varieties of lettuce retained significantly different volumes of wastewater ($p < 0.01$). No statistical differences ($p > 0.01$) were detected between *E. coli* counts obtained from different parts of lettuce, nor between the direct and indirect enumeration methods. Statistically significant linear relationships were derived relating the *E. coli* concentration of the wastewater in which the lettuces were submerged to the subsequent *E. coli* count on each variety the lettuce.

**Key words** | health risk, leaf morphology, lettuce, microbial risk, wastewater irrigation

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## INTRODUCTION

Worldwide, wastewater irrigation of crops is being applied increasingly in agriculture due to pressures associated with population growth and the shortage of freshwater resources. One of the major public health concerns from this practice is the human health risk from exposure to pathogens associated with the consumption of wastewater-irrigated crops, particularly salad crops, which are generally consumed raw. The consumption of lettuce has been linked to several outbreaks of foodborne diseases (Brandl & Amundson 2008; Barker-Reid *et al.* 2009). In an effort to minimise the adverse health effects from human exposure to pathogens associated with wastewater reuse in agriculture, the World Health Organization (WHO) published the third edition of the guidelines for the safe use of wastewater, excreta and greywater in 2006 (WHO 2006). The guidelines offer multiple approaches to risk management to meet the health-based target for the burden of waterborne disease, $\leq 10^{-6}$ disability adjusted life years, associated with working in wastewater-irrigated farms, or consuming wastewater-irrigated crops. In this guideline, a quantitative microbial risk assessment (QMRA) approach (Haas *et al.* 1999) was used to estimate the health risk from wastewater irrigation. In brief, QMRA translates the exposure of consumers to pathogens under a specific set of conditions (exposure scenarios) to the probabilities of infection by applying four steps, namely: hazard identification, dose-response assessment, exposure assessment and risk characterisation. Although QMRA could be an effective tool for health risk estimation,

doi: 10.2166/wh.2016.145

the challenges of using this tool include the lack of data or the poor quality of data available for inclusion in the estimation of the risk. Many studies have applied QMRA to the consumption of wastewater-irrigated salad crops, and lettuce, consumed worldwide, has been widely used to estimate this health risk.

In most of the QMRA studies, including the current WHO guidelines, the level of microbial contamination of the crops was estimated using exposure assessments derived from the water retained on the crops' surface, accepting the assumption that any microorganisms contained in the residual wastewater will be retained on the vegetable surfaces, even after the wastewater has evaporated (Shuval *et al.* 1997; Petterson *et al.* 2001; Hamilton *et al.* 2006; Mara *et al.* 2007). Based on this assumption, it is important to identify the water retention in various morphological varieties of lettuce since it has only been determined for one type of lettuce (long leaf lettuce) by Shuval *et al.* (1997) who determined water retention from the difference in weight following submersion of lettuce in a bucket of water. There is a variety of lettuce cultivars, Iceberg, Cos and Oak leaf lettuce, with different morphology, grown and commonly consumed across the world (Van Treuren & Van Hintum 2009; Mou 2012). At present, there is limited information about water retention in various varieties of lettuce, which could be useful for estimating risk using QMRA. Furthermore, there are few studies that attempt to determine directly the numbers of microorganisms retained on the plants' surfaces to estimate the risk, rather than using the volume of wastewater, with a known concentration of microorganisms of concern, retained on the crops' surface to estimate numbers (Bastos *et al.* 2008; Aiello *et al.* 2012; Forslund *et al.* 2012). Nevertheless, Mok & Hamilton (2014) argued that this direct method was not flexible for modelling multiple scenarios compared to the water retention method, as it will only allow modelling on a particular set of conditions. However, enumeration directly from the surface of crops is the standard method for the microbiological examination of fresh fruits and vegetables used by food standard regulatory agencies (Food Standards Australia New Zealand (FSANZ) 2001; United States Food & Drug Administration (USFDA) 2003).

There is no clear evidence if the numbers estimated from the water retention on the surface of plants following submersion are comparable to the number of microorganisms quantified directly from the plants' surface. The aims of this study were: firstly, to determine wastewater retention volumes for three different varieties of lettuce (Iceberg, Cos and Oak leaf); secondly, after submersion, to compare the *Escherichia coli* concentration on composite samples and samples of outer and inner leaves from the three different varieties of lettuce; and, finally, to determine the effect of microbial wastewater quality on the contamination of *E. coli* on lettuce leaves, and to compare the direct enumeration of *E. coli* on lettuce leaves with the indirect method, which estimates contamination using the *E. coli* concentration and the volume of wastewater retained.

## METHODS

### Sample selection

#### Lettuce varieties

Three varieties of lettuce, Iceberg, Cos and Oak leaf lettuce (Figure 1), which are widely consumed, were selected for this study as they have different leaf structures, which could potentially affect water retention. Lettuce samples were bought from local supermarkets in Adelaide, South Australia. Each lettuce was contained individually in a clear polythene freezer bag, and transported, chilled in a freezer box, to Environmental Health Laboratories, Flinders University for analysis. Oak leaf lettuces normally came with the roots attached, which were removed aseptically with a sterile knife (wiped with 70% ethanol and flamed).

### Wastewater samples

In order to determine the effect of microbial wastewater quality on the contamination of lettuce, four different target concentrations of *E. coli* in wastewater were selected; 10, $10^2$, $10^3$ and $10^4$ *E. coli* most probable number (MPN)/100 mL. The wastewater samples (40 L) were collected at different points from the wastewater stabilisation ponds (WSP), Mt Barker wastewater treatment plant, South Australia. The concentration of *E. coli* in the various wastewaters used in the experiments and the collection points are shown in Table 1.

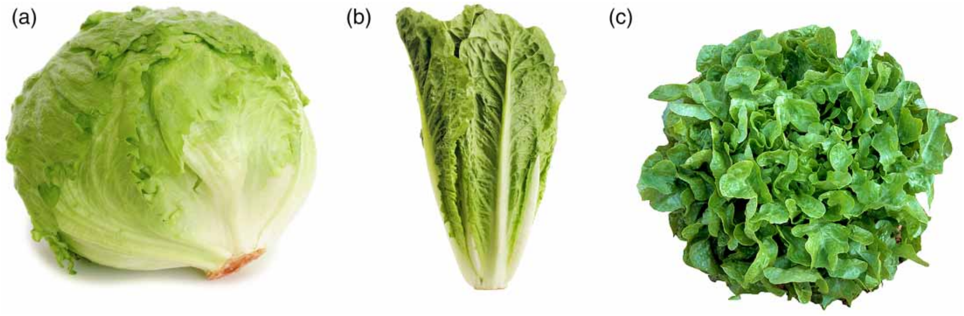

**Figure 1** | The three varieties of lettuce used in this study. (a) Iceberg (http://www.samsclub.com/sams/taylor-farms-iceberg-lettuce-2-heads/133615.ip), (b) Cos (http://www.samsclub.com/sams/romaine-hearts-6-ct/prod1941521.ip), (c) Oak leaf (http://montecitourbanfarms.com/shop/salanova-green-oakleaf-lettuce/#prettyPhoto).

**Table 1** | The concentration of *E. coli* (MPN/100 mL) in wastewater samples from various sources

| Target concentration | Actual concentration | Wastewater source (Mt Barker[a]) |
|---|---|---|
| 10 | 6.3 | The outlet of wastewater DAF[b]plant. |
| $10^2$ | 75.9 | Final pond in the WSP series |
| $10^3$ | 1,299.7 | The dilution of the first facultative pond: with the outlet water from the DAF[b] (10: 1) |
| $10^4$ | 27,550 | Wastewater from the first facultative pond |

[a] Mt Barker Community Wastewater Treatment Plant, South Australia.
[b] DAF: dissolved air flotation.

## Wastewater retention

To determine the influence of lettuce cultivars on water retention and *E. coli* contamination, lettuces were contaminated with wastewater in the laboratory using the bucket submersion technique (Hawley 2012), which was adapted from Shuval *et al.* (1997). Subsequently, this method was shown to be a surrogate for the assessment of lettuce spray-irrigated with wastewater in the field (Makkaew *et al.* 2016). A 10 L plastic bucket, placed on a larger aluminium tray to contain spillage, was used for lettuce submersion. The whole lettuces were submerged, individually, upside down into the bucket for 20 s. Then, each submersed lettuce was held above the bucket and gently flicked right to left, left to right, and up and down, eight times each way. This sequence was performed four times, the lettuce was then held above the water for 20 s after the last submersion to drain surplus water. Six samples of each type of lettuce were contaminated using this procedure for each of four experiments using wastewaters with the different concentrations of *E. coli* (Table 1).

This bucket submersion method was applied to 72 lettuces (three varieties of lettuce, six samples per lettuce variety with four different *E. coli* concentrations in wastewater). Each lettuce was weighed individually in an aluminium foil lined plastic bowl before and after submersion. The volume of water retained was calculated using Equation (1).

$$W_r = W_a - W_b \qquad (1)$$

where $W_r$ was the volume of water retained (mL/g lettuce), $W_a$ was the weight (g) of lettuce after submersion and $W_b$ was the initial weight (g) of lettuce before submersion. Then, the volume of water retained was calculated (Equation (2)) and expressed as water retained per 100 g of lettuce based on the current guidelines (Shuval *et al.* 1997; WHO 2006). The volume of water retained was calculated by:

$$W_{r100} = \left(\frac{W_r}{W_b}\right) * 100\,\text{g Lettuce} \qquad (2)$$

## Enumeration of *E. coli*

### Indirect method

The *E. coli* content of the respective wastewater in which the lettuces were submerged was determined using the

Colilert®-18 MPN method (IDDEX Laboratories). The number of *E. coli* on lettuce leaves was calculated from the *E. coli* concentration of the wastewater and the volume retained by the lettuce using Equation (3).

$$\frac{\left[\left(\frac{E.coli\ \text{concentration}}{100\,\text{mL wastewater}}\right)*\left(\frac{\text{Volume of water retained}}{100\,\text{g of lettuce}}\right)\right]}{100} \tag{3}$$

### Direct method

Following wastewater submersion, the lettuce was dissected into two components each comprising 3–4 outer leaves and 3–4 inner leaves in order to determine the *E. coli* concentration retained on lettuce leaves from different leaf locations. The outer and inner leaf samples were cut aseptically into 25 g. A second wastewater submersed lettuce, of the same type, was cut into quarters and then aseptically dissected into 25 g to include all parts of the lettuce leaves; this was designated the composite leaf sample. This experiment was conducted using three lettuce varieties each at four different *E. coli* concentrations in wastewater. The respective dissected leaf parts were analysed in triplicate. Each 25 g lettuce sample was added to a stomacher bag containing 225 mL, 0.1% sterile buffered peptone water and homogenised using a stomacher (Model 2X (IDEXX)) for 1 minute. Afterwards, 100 mL of suspension from the homogenate was collected into a 120 mL sterile tube and enumerated for *E. coli* using the Colilert®-18 MPN method. The results were expressed as the MPN of *E. coli*/100 g of lettuce.

### Statistical analysis

The difference in wastewater retained, and recovered *E. coli* between different varieties and parts of lettuce was analysed by using two-way analysis of variance together with the Bonferroni *post-hoc* test. The difference in *E. coli* concentration between the direct and indirect method of enumeration was analysed using the Paired-T test. The relationship of the *E. coli* concentration between the *E. coli* concentration of the wastewater and lettuces following submersion was analysed using linear regression. All statistical analyses were performed using SPSS (PASW Statistics 18) with a confidence level of 95%.

## RESULTS

### Water retention in three varieties of lettuce

The mean volume of wastewater retained by the three lettuce varieties using the indirect method of determination is shown in Figure 2. There was a statistically significant difference in the volume of wastewater retained by the three different varieties of lettuce ($p < 0.01$). It can be seen that Oak leaf lettuce retained the highest volume (42.9 ± 4.9 mL/100 g), following by Cos (22.6 ± 4.8 mL/100 g) and Iceberg (15 ± 4.6 mL/100 g).

### *E. coli* retained by varieties and parts of lettuce

*E. coli* retained by parts of three different varieties of lettuce after submersion in wastewaters of differing *E. coli* concentrations, enumerated using the direct method, is shown in Figures 3–5. No *E. coli* was detected in any lettuce samples following submersion in wastewater containing 6.3 *E. coli* MPN/100 mL. There were, however, statistically significant differences ($p < 0.01$) in *E. coli* concentration retained between Iceberg and both the Cos and Oak leaf varieties, with Iceberg retaining significantly less *E. coli* following submersion in wastewaters containing 75.9, 1,299.7 and 27,550 *E. coli* MPN/100 mL. In addition, there was no statistically significant difference between outer and inner leaves and

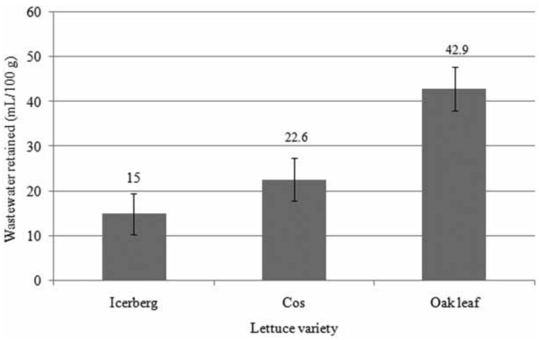

**Figure 2** | Wastewater retained (mL/100 g) on the leaves of Iceberg, Cos and Oak leaf lettuce ($n = 24$ for each lettuce cultivar).

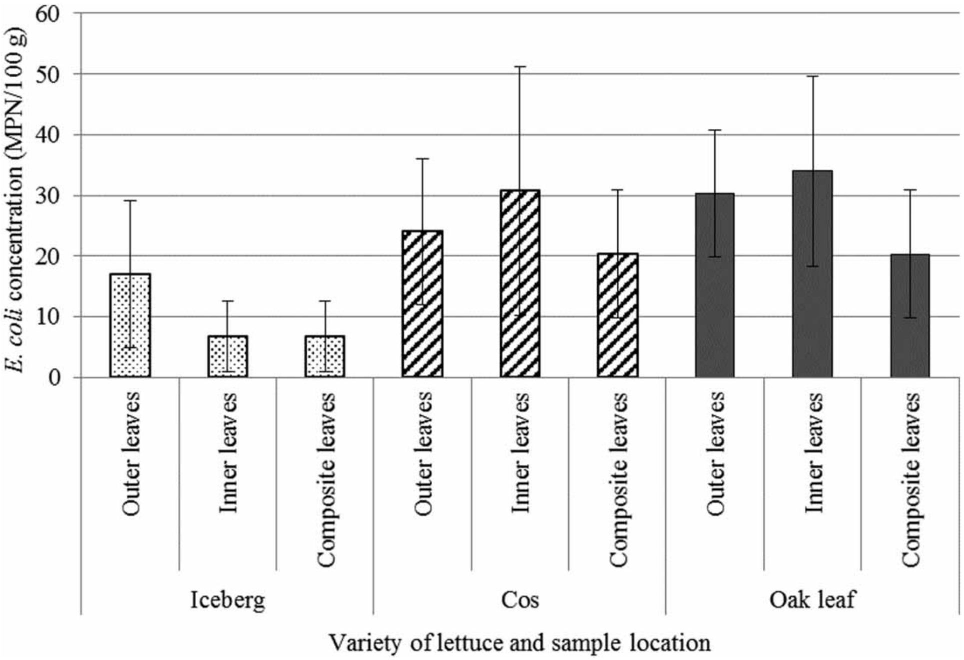

**Figure 3** | *E. coli* enumerated in outer and inner leaves and a composite sample of Iceberg, Cos and Oak leaf submersed in wastewater containing 75.9 *E. coli* MPN/100 mL.

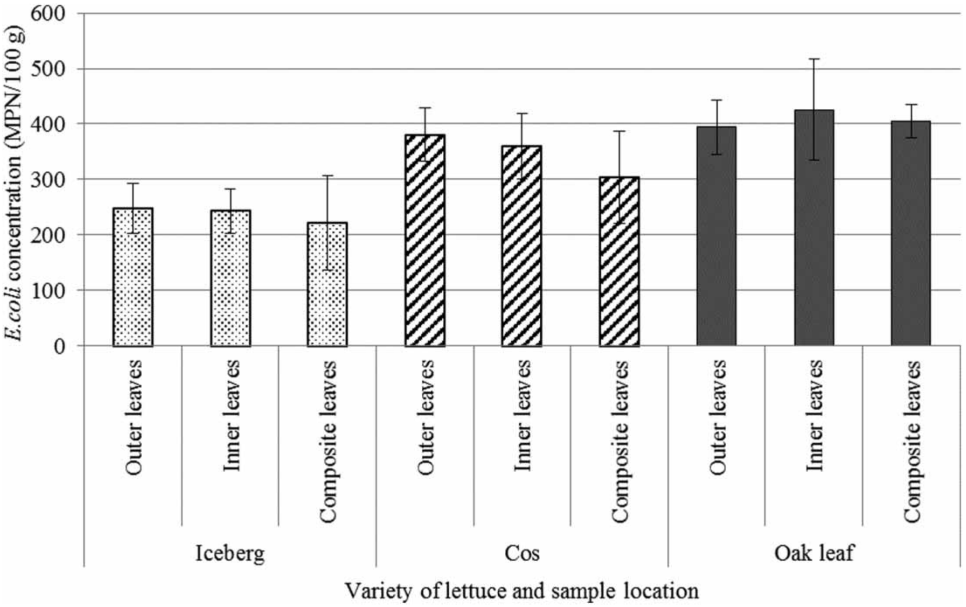

**Figure 4** | *E. coli* enumerated in outer and inner leaves and a composite sample of Iceberg, Cos and Oak leaf submersed in wastewater containing 1,299.7 *E. coli* MPN/100 mL.

the composite leaf samples ($p > 0.01$) following submersion in the wastewaters with *E. coli* concentrations of 75.9 and 1,299.7 *E. coli* MPN/100 mL (Figures 3 and 4). However, the location of the lettuce leaves becomes an important factor when submersion is in wastewater with an *E. coli* concentration of 27,550 *E. coli* MPN/100 mL, when there were

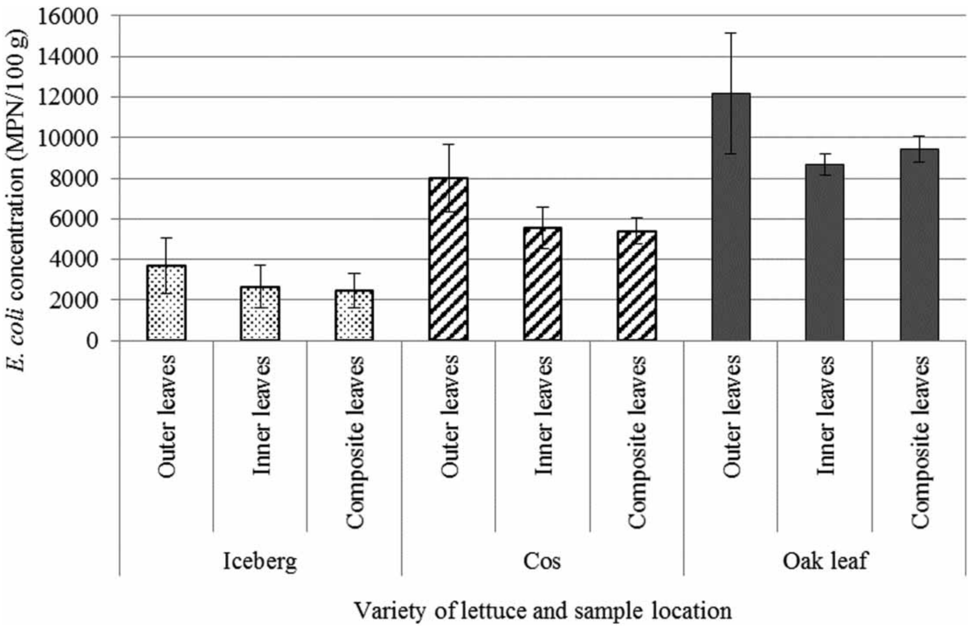

**Figure 5** | *E. coli* enumerated in outer and inner leaves and a composite sample of Iceberg, Cos and Oak leaf submersed in wastewater containing 27,550 *E. coli* MPN/100 mL.

statistically significant differences in *E. coli* concentration between outer and inner leaves and the composite samples ($p < 0.01$; Figure 5).

## The comparison of *E. coli* concentration enumerated by direct and indirect method

The direct method (quantified from the lettuce sampled after wastewater submersion) and indirect method (estimate of the concentration based on the retained wastewater on the lettuce leaf surfaces as described in Equation (3)) for determining the retention of *E. coli* were compared. The arithmetic mean *E. coli* count of the three composite samples from each lettuce type was used to represent the concentration of *E. coli* enumerated by the direct method. The composite samples were used in this comparison as they were considered to better represent the number of *E. coli* enumerated using the standard method for the microbiological examination of fresh produce employed by food regulatory agencies (FSANZ 2001; USFDA 2003). The data are shown in Table 2. There were no statistically significant differences in *E. coli* concentration for all three varieties of lettuce between the direct and indirect method of enumeration ($p > 0.01$).

**Table 2** | The comparison of *E. coli* concentration quantified by the direct method, enumerated from a 25 g composite sample of lettuce, and the indirect method, estimated from the *E. coli* concentration of the wastewater and the volume retained by the lettuce

| Lettuce varieties | Microbial wastewater quality (*E. coli* MPN/100 mL) | *E. coli* concentration (MPN/100 g) (Mean ± S.D.) Direct method | Indirect method |
|---|---|---|---|
| Iceberg | 75.9 ($n = 3$) | 6.7 ± 5.8 | 10.2 ± 3.3 |
| | 1,299.7 ($n = 3$) | 221.3 ± 84.9 | 237.4 ± 57.4 |
| | 27,550 ($n = 3$) | 2,470.7 ± 848.8 | 3,278.5 ± 600.4 |
| Cos | 75.9 ($n = 3$) | 20.3 ± 10.5 | 17.1 ± 2.9 |
| | 1,299.7 ($n = 3$) | 303.3 ± 83.1 | 262.5 ± 15.8 |
| | 27,550 ($n = 3$) | 5,395.7 ± 652.9 | 5,234.5 ± 1,360.6 |
| Oak leaf | 75.9 ($n = 3$) | 20.3 ± 10.5 | 32.0 ± 1.6 |
| | 1,299.7 ($n = 3$) | 405.0 ± 29.8 | 510.8 ± 20.4 |
| | 27,550 ($n = 3$) | 9,424.0 ± 658.2 | 11,470.0 ± 937.2 |

## The relationship of microbial quality between lettuce and wastewater

The *E. coli* counts enumerated from composite leaves of the respective lettuces were used to determine the relationship between the *E. coli* concentrations of the wastewater in which they were submersed. The *E. coli* concentration on lettuces was significantly ($p < 0.05$) related to *E. coli* concentration of the wastewater (Figure 6).

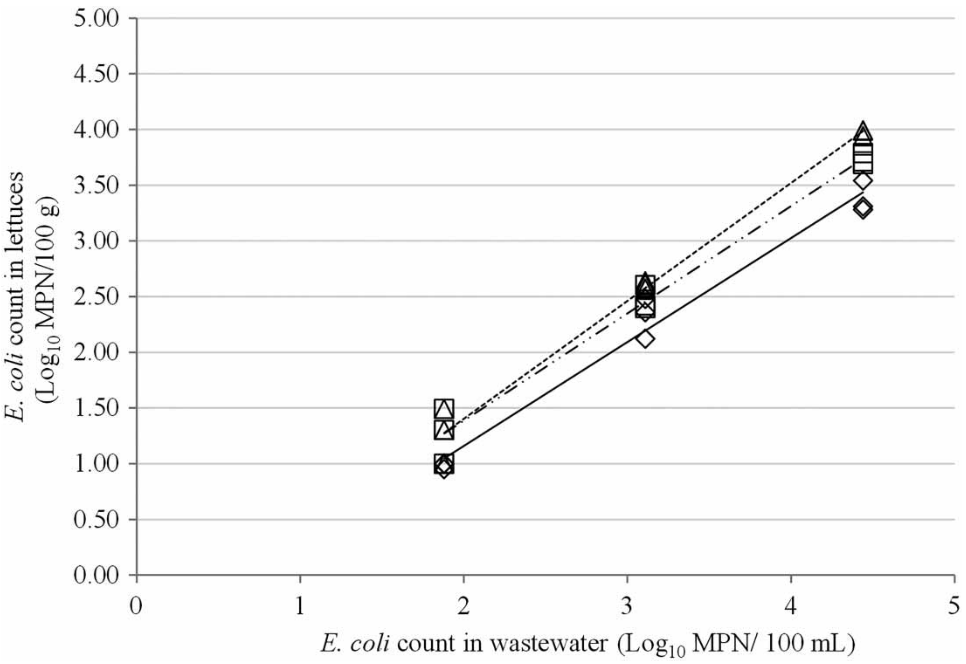

**Figure 6** | The linear regression of *E. coli* count (log$_{10}$ *E. coli* MPN/100 g) recovered from composite leaf samples of Iceberg (◇, —, Equation (6)), Cos (□, —··— Equation (5)) and Oak leaf (△, ----- Equation (4)) lettuce and the *E. coli* count in the wastewater in which they were submersed (log$_{10}$ *E. coli* MPN/100 mL).

## DISCUSSION

Wastewater irrigation in agriculture is an emerging public health risk as it is becoming more widely used worldwide, but there are still knowledge gaps with regard to assessing the health risk from consuming crops irrigated with wastewater. Shuval *et al.* (1997) were the first to estimate the health risk based on the captured volume of water on the surface of lettuce leaves, which was subsequently used by a number of other studies (Petterson *et al.* 2001; Hamilton *et al.* 2006; Mara *et al.* 2007). The study reported here, determined the volume of wastewater retained on three different varieties of lettuce (Iceberg, Cos and Oak leaf) since there was limited data available regarding water retention by different lettuce varieties. The results of this study showed that the different lettuce varieties retain differing amounts of wastewater; Oak leaf retained significantly more wastewater per 100 g of lettuce than either Cos or Iceberg. The differences are likely due to differences in leaf morphology between different varieties of lettuce and consequently, the relative surface area exposed to submersed wastewater. Iceberg has tightly compacted, overlapping leaves, while Oak leaf has a loose head with broad and elongated rosette leaves (Křístková *et al.* 2008; Hawley 2012). These leaf structures affect the wastewater retention on the lettuce leaf surfaces. The compact leaves of the Iceberg variety could prevent the water getting inside the heart of the lettuce; whereas the more open leaf varieties like Oak leaf retain more of the water. In addition, crop leaves also have some properties, which could potentially influence the wastewater retention on the leaf surfaces. Hunter *et al.* (2010) found that Iceberg lettuce leaves had slightly more wax content than other lettuce varieties, causing the water to roll off the surface immediately instead of being adsorbed into the leaves (Neinhuis & Barthlott 1997).

In this study, the retained wastewater volume of the three lettuce varieties (15± 4.6, 22.6± 4.8 and 42.9± 4.9 mL/100 g for Iceberg, Cos and Oak leaf lettuce, respectively) was higher than the mean volume (10.8 mL/100 g) reported by Shuval *et al.* (1997), which has been widely used in subsequent QMRAs (Petterson *et al.* 2001; Hamilton *et al.* 2006; Mara *et al.* 2007). The variability associated with the water volumes captured by lettuce and used in stochastic (probabilistic) QMRAs has been variously addressed by assigning a normal probability density function (Hamilton *et al.* 2006) to the point estimate originally determined by Shuval *et al.* (1997). Whereas Mara *et al.* (2007) incorporated a linear range (10–15 mL/100 g) around the value of 10.8 mL. There are potentially some factors explaining the difference in the retained wastewater volume; the varieties of lettuce used in this experiment were different from the long leaf lettuces used by Shuval *et al.* (1997). The sample size also differed, only 12 long leaf lettuces were used to determine the captured water in the study by Shuval *et al.* (1997), while the sample size of the study reported here was 24 plants per lettuce variety. The water applied could also be another factor influencing the amount of retained water on the crop surfaces. Lettuces were submersed into wastewater in this study, whereas there was no description of the water type used in the experiment conducted by Shuval *et al.* (1997). Hawley (2012) compared the amount of water retention in lettuce following submersion in either potable water or domestic wastewater from a waste stabilisation pond. She noted that the volume of retained water was greater when lettuces were submersed in wastewater. Suspended solids are the major component of organic contaminants in domestic wastewater (Mckinney 2004). These could adhere to the lettuce leaf surfaces, resulting in the greater weight gain, interpreted as the volume of water retained, compared with when potable water was used in the experiments.

Mok & Hamilton (2014) investigated the captured water volume of some Asian vegetables as well as Oak leaf lettuce. The captured volume of Oak leaf lettuce in their study, 1 mL/100 g was far less than from our study, 42.9 mL/100 g. This difference could possibly be explained by the difference in experimental procedures used to examine the water retained on the crops' surfaces. Mok & Hamilton (2014) collected a hundred Oak leaf lettuces from a field irrigated with freshwater by overhead sprinklers, to determine the captured volume by weight differential before and after spinning and drying with a paper towel, while a laboratory submersion technique, as described above, was used in our study. The bucket submersion technique exposed a greater surface area of the crops to the wastewater in comparison to irrigation by overhead sprinklers where the water falls as droplets onto the surface of the crop. In addition, the weighing protocol was also different between these two studies. In our laboratory study the whole lettuce was submersed into wastewater contained within a bucket, excess water was removed using a well-defined 'shaking' protocol and the captured volume was calculated by difference in weight before and after submersion. In contrast, in the study reported by Mok & Hamilton (2014) significant manipulation of the samples occurred before the water retention value was determined. The Oak leaf lettuces were cut from the field, transported to a laboratory, weighed, cut into small pieces before being spun, and weighed. The water retained on crops' surfaces was potentially lost during transportation, leaf dissection and spinning, resulting in a much-reduced value for water retention than that reported here (42.9 mL/100 g) or the 10.8 mL/100 g reported by Shuval *et al.* (1997). In addition, the volume of wastewater captured by the lettuce reported by Mok & Hamilton (2014) was determined by the weight lost before and after spinning, whereas our study determined wastewater retention from the difference in the weight of the lettuce pre- and post-submersion. The sample manipulations conducted by Mok & Hamilton (2014), could, arguably, be considered to more accurately reflect the microbial contamination where transport and handling is more intense and consequently reduces both the wastewater retained and the associated pathogens. The submersion technique reported here might, however, be the more valid approach where worse-case scenario, rapid risk assessment for use in exposure models in QMRA studies is required.

The morphology of crops' leaves also affects the microbial contamination; in our study *E. coli* were detected in larger numbers in Oak leaf compared to Cos and Iceberg. Dense foliage crops have also been observed to be more contaminated by parasites. Amahmid *et al.* (1999) reported greater numbers of *Giardia* cysts and *Ascaris* eggs detected in coriander and mint compared to carrot and radish when irrigated with raw wastewater because the larger surface area of the herbs could capture more irrigated wastewater.

Microbial contamination might plausibly be influenced by the relative exposure of the leaves to the irrigating wastewater and, further, by the location from which the sample was obtained for analysis. There was no statistically significant difference ($p > 0.01$) amongst outer and inner leaves and composite samples of leaves following submersion in wastewaters with *E. coli* concentrations of 75.9 and 1,299.7 *E. coli* MPN/100 mL. However, when submersed in wastewater with an *E. coli* concentration of 27,550 *E. coli* MPN/100 mL, the number of *E. coli* was higher on the outer leaves than on both inner and composite leaf samples of all three varieties of lettuce studied. A similar result was observed by Oliveira *et al.* (2012) under a field condition where Cos lettuces were exposed to water contaminated with $10^7$ *E. coli* O157:H7 CFU/mL, manually applied by surface or hand spray irrigation. They reported more contamination on the outer leaves than the inner ones, presumably since the outer leaves were more exposed to the contaminate spray and were potentially in direct contact with contaminated soil and water. Therefore, a possible risk mitigation approach for food regulators may be to recommend that consumers discard the outer leaves of lettuce before washing in order to reduce the risk posed by contaminating pathogenic microorganisms.

Uniquely, the study reported here compared both direct (sampling the leaves) and indirect (water retention) methods for determining or estimating microbial contamination of lettuce following submersion in wastewater contaminated with differing concentrations of *E. coli*. An important finding was that there was no statistical difference ($p > 0.01$), irrespective of the lettuce variety, in the *E. coli* concentration recovered from the lettuce using the direct method of determination and that estimated indirectly from the weight of water retained following submersion. The indirect estimation approach was used in many studies (Shuval *et al.* 1997; Petterson *et al.* 2001; Hamilton *et al.* 2006; Mara *et al.* 2007), few have used the direct method to determine the numbers of microorganisms on the crops' surfaces to estimate the risk associated with consumption (Bastos *et al.* 2008; Aiello *et al.* 2012; Forslund *et al.* 2012). Our findings suggest that the results from studies using either the direct or the indirect methods are broadly comparable. In this study the direct and indirect methods yielded equally valid data for estimating the health risk from consumption of wastewater-irrigated salad crops. However, it is emerging that human pathogens such as *E. coli* OH157:H7 and *Salmonella enterica* are able to access and, given sufficient available nutrients, grow on interior surfaces of plants (Brandl & Amundson 2008; Saldana *et al.* 2011). The internalisation of human pathogens is an additional hazard, which needs consideration in future risk assessments of wastewater-irrigated salad crops. Inclusion of the risk from internalisation and growth of human pathogens requires that the direct enumeration of microorganisms from the leaves of wastewater-irrigated salad crops should in future be the preferred method for assessing contamination by food regulatory agencies.

Our study shows that *E. coli* counts on lettuces were significantly ($p < 0.05$) related with the *E. coli* concentration in the wastewater in which they were submersed. High concentration of *E. coli* in irrigating wastewater results in high contamination of wastewater-irrigated crops. Furthermore, the relationship between the *E. coli* concentration in the wastewater and in composite lettuce samples following submersion was shown to be linear; the *E. coli* concentration in lettuce could be predicted from the *E. coli* concentration in wastewater (Equations (4)–(6)).

Oakleaf

$$\log_{10}(E.coli\ \text{lettuce}) = 1.06\ \log_{10}(E.coli\ \text{wastewater}) -0.71(n = 9;\ R^2 = 0.988) \quad (4)$$

Cos

$$\log_{10}(E.coli\ \text{lettuce}) = 0.93\ \log_{10}(E.coli\ \text{wastewater}) -0.53(n = 9;\ R^2 = 0.983) \quad (5)$$

Iceberg

$$\log_{10}(E.coli\ \text{lettuce}) = 0.93\ \log_{10}(E.coli\ \text{wastewater}) -0.70(n = 9;\ R^2 = 0.980) \quad (6)$$

where

*E. coli* wastewater = *E. coli* MPN/100 mL
*E. coli* lettuce = *E. coli* MPN/100 g

Bastos *et al.* (2008) also derived similar equations, using results from field studies, relating wastewater quality to contamination of salad crops. Two equations were derived to estimate the *E. coli* concentration of high-growing crops (kale and green pepper) and low-growing crops (lettuce, spinach, and arugula; Equation (7)) at harvest.

Low-growing crops:

$$\log_{10}(E.coli\ \text{crops}) = 0.83\ \log_{10}(E.coli\ \text{wastewater}) - 0.73 \quad (7)$$

**Table 3** | The predicted *E. coli* concentration on lettuce irrigated with wastewater containing 10, $10^2$, $10^3$ and $10^4$ *E. coli* MPN/100 mL calculated using Equations (4)–(6) (this study) and Equation (7) (Bastos *et al.* 2008)

| | **Predicted bacterial quality of lettuce (*E. coli* MPN/g)** | | | |
|---|---|---|---|---|
| **Wastewater qualities (*E. coli* MPN/100 mL)** | **Equation (4)** | **Equation (5)** | **Equation (6)** | **Equation (7)** |
| 10 | 0.02 | 0.03 | 0.02 | 1.26 |
| $10^2$ | 0.26 | 0.21 | 0.14 | 8.51 |
| $10^3$ | 2.95 | 1.82 | 1.23 | 57.54 |
| $10^4$ | 33.88 | 15.48 | 10.47 | 398.05 |

where

*E. coli* wastewater = *E. coli* MPN/100 mL
*E. coli* crops = *E. coli* MPN/g

Although the equation of Bastos *et al.* (2008) was different to those reported here, there was no statistical difference (Independent-Samples T Test, $p > 0.05$; Equations (7) and (4), (7) and (5), and (7) and (6) in the predicted microbial quality of crops (Table 3) modelled using irrigation wastewater qualities of 10, $10^2$, $10^3$ and $10^4$ *E. coli* MPN/100 mL. The *E. coli* counts on the lettuce predicted from Equations (4)–(6) were converted from *E. coli* MPN/100 g to *E. coli* MPN/g in order to be consistent with Equation (7) of Bastos *et al.* (2008).

However, Equation (7) (Bastos *et al.* 2008) was derived from low-growing salad crops data, including spinach, arugula, and lettuce (no cultivar defined in their study), while equations reported here were derived from the data obtained from three different varieties of lettuce. The equations derived from this study, as well as the equation derived from Bastos *et al.* (2008), could be used for the preliminary assessment of microbial risk in salad crops when the *E. coli* concentration of the irrigating wastewater is known.

## CONCLUSIONS

In summary, the laboratory based experiment using the bucket submersion technique as a surrogate for field, spray irrigation, showed that the different cultivars of lettuce had different wastewater retention capabilities; the volume of wastewater retained by Oak leaf was greater than that retained by either Cos or Iceberg lettuce. There was no statistical difference in the *E. coli* count obtained from outer, inner and composite samples of leaves following submersion in wastewaters with *E. coli* concentrations of $10^2$ and $10^3$ *E. coli* MPN/100 mL. However, the *E. coli* count was higher on the outer leaves than on either inner or composite leaf samples of lettuce following submersion in wastewater with an *E. coli* concentration of $10^4$ *E. coli* MPN/100 mL. Equations were derived which described the statistically significant linear relationship between the *E. coli* concentration of the wastewater and the subsequent *E. coli* count obtained from composite leaf samples following submersion. Uniquely, this study was the first to confirm that using the direct enumeration technique, where *E. coli* was enumerated on the leaves after submersion in wastewater was comparable with the indirect technique, where the *E. coli* concentration was estimated from the volume of wastewater retained by the lettuce and the *E. coli* concentration of the wastewater. This finding will be useful for conducting QMRA associated with the consumption of wastewater-irrigated salad crops.

## ACKNOWLEDGEMENTS

This study was supported by funding from School of the Environment, Faculty of Science and Engineering, Flinders University, South Australia and the Flinders-Hunan Seed Funding Project. Prasert Makkaew is also grateful to the Royal Thai Government, Thailand for granting a PhD scholarship. Thanks is also given to Mt Barker District Council for access to their wastewater treatment facilities.

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First received 17 May 2016; accepted in revised form 16 October 2016. Available online 9 December 2016