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Frogs host faecal bacteria typically associated with humans

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Abstract

Tree frogs commonly access drinking water tanks, which may have human health implications. Although amphibians might not be expected to host mammalian faecal indicator bacteria (FIB), it is possible that they may have human FIB on their skin after exposure to human waste. We collected faeces and skin wash from green tree frogs *(Litoria caerulea*) from a natural environment, a suburban site, and a suburban site near a creek occasionally contaminated with sewage effluent. We used molecular techniques to test for FIB that are routinely used to indicate human faecal contamination. Enterococci colonies were isolated from both faecal and skin wash samples, and specific markers (*E. faecium* and *B. thetaiotamicron*) were found in frog faeces, demonstrating that these markers are not human or mammalian-specific. *B. thetaiotamicron* was detected in frogs from both natural and urban sites, but *E. faecium* was only associated with the sewage impacted site.

Key words

Animals; drinking water; sewage; water quality.

Introduction

The safety of drinking water is a fundamental public health issue, particularly in the tropics where high temperatures are conducive to bacterial growth. One of the standard tests for water quality is to measure levels of enterococci because they are considered to be reliable mammalian-specific faecal indicators (National Health and Medical Research Council 2008). Tests for *E. faecium* are considered to be more specific for human faeces than the genus level test (Scott *et al*. 2005), however dogs and seals have also been shown to be positive for this species (Layton *et al.* 2009). The public health implication of detecting *E. faecium* in non-human hosts is not well understood, and one solution has been to advocate multiple tests that target a range of microbes generally associated with human faeces (Neave *et al.* 2014). As a result, we now have more targets for human FIB tests including *Bacteroides thetaiotamicron* that is widely thought to be human-specific (Teng *et al*. 2004). The predominance of *B. thetaiotaomicron* in human feces makes it a worthy candidate as a marker of human fecal matter. Although these targets are not necessarily human specific, it has been assumed that they are

mammalian specific. Since the health risk for contact with human feces is generally higher than that with non-human feces, this is an important development in terms of identifying human fecal matter (Carson et al. 2005).

Our interest in non-mammalian hosts was sparked by an anecdotal report that residents of a remote community in the wet-dry tropics of northern Australia were experiencing symptoms of gastroenteritis and a link was made to the quality of water in domestic rainwater tanks. Green tree frogs (*Litoria caerulea*) commonly inhabit these water tanks in northern tropical Australia, leading us to investigate whether in fact these frogs can host bacteria typically associated with human faeces. *L. caerulea* is an important source of yeasts in water storage reservoirs in areas where the animal is endemic (Sammon et al., 2009; 2010), so if frogs do host faecal bacteria that are commonly thought to be human-specific, this has further implications for human health.

If these bacteria were present, we also wanted to determine whether the bacteria were in frog faeces, on their skin, or both. If frogs carry these bacteria on their skin, they might contaminate drinking water for human consumption even without defecating in the water supply. Furthermore, if

frogs are hosts for indicator bacteria, we wanted to determine if this occurs in natural habitats, or whether it is only found in close association with humans. If the frogs are contaminated from humans, does this result from routine exposure (typical suburban associations, including possible contact with toilets), or is it a result of direct exposure to sewage outflows? To answer these questions, we collected faeces and skin wash from *Litoria caerulea* from a suburban area (suburban), a suburban area next to a creek that is known to sometimes receive sewage effluent (contaminated), and a natural (reference) environment. We then used molecular techniques to test for the presence of bacteria that are routinely used to detect human faecal contamination – *Enterococcus faecium* and *Bacteroides thetaiotamicron*.

Materials and methods

Sites

Green tree frogs were sampled from three sites in the vicinity of Darwin, Northern Territory, Australia. The suburb of Ludmilla is an area in which frogs inhabit suburban gardens adjacent to Ludmilla Creek, which receives periodic

sewage discharges (referred to as potentially "contaminated") (Hind, 2014). The suburb of Brinkin represents an area in which frogs inhabit suburban gardens that are not near sewage outlets (referred to as "suburban"). The area near Mickett Creek (MC) on the outskirts of Darwin represents a natural or "reference" site of approximately 50 ha with natural vegetation typical of wet–dry tropical savannah habitat. Here, the frogs live in trees instead of being associated with buildings.

Frogs

Ten green tree frogs (*Litoria caerulea*) were captured by hand (using fresh, disposable gloves) from both Ludmilla Creek and Brinkin, but despite multiple trips and intense searching we were only able to find and capture three frogs at Mickett Creek. Soil samples from near each capture location were also collected. Once the frogs were brought into the lab, skin wash was collected as follows.

One researcher slowly poured 50 mL of sterile distilled water over the frog as another researcher held the frog and gently massaged its skin. This washing is hereafter referred to as skin wash (SW). Each frog was then placed in a ventilated plastic container, and placed in a constant temperature room at 30°C. The plastic containers were tilted so that the frogs had access to water

at one end, but the other end was dry. The frogs were checked every few hours, and faecal samples were removed from the containers as soon as they were found. Once a frog defecated, it was released at night at its original capture location.

Enterococci enrichment

Faeces were collected and suspended in sterile phosphate buffered saline (PBS) at a ratio of 1:10, shaken for 10 min at room temperature and allowed to settle. A 200 µ L sample of the faecal suspension was plated onto enterococci-specific mEI Agar overlaid with a sterile, white, gridded 47 mm diameter, 0.45 µm pore size membrane filter, and plates were then processed according to standard methods (USEPA 2006). SW was collected as described above, and 25 mL samples were filtered under suction through a membrane filter and enriched for enterococci as described for faeces. A 10 g aliquot of each soil sample was mixed with 60 mL PBS, shaken by hand for 2 min and allowed to settle for 30 s. This eluant was decanted into another bottle and 40 mL PBS was added to the original 10 g of soil, shaken and settled. The two eluants were mixed and filtered under suction through a membrane filter. The filter was transferred to mEI media and enriched for enterococci as described for faeces.

Enterococci DNA extraction

After incubation of faeces, SW and soil plates, colonies approximately 0.5 mm in diameter with a blue halo were transferred to 4 mL Tryptic Soy Broth (TSB), vortexed and incubated at 41°C for 3 h in a shaker. The TSB and filter was then centrifuged for 5 min @ 4500 g. DNA was extracted from the resulting pellet using the MoBio Microbe® DNA extraction kit according to the manufacturer's instructions. The purified DNA was taken up in 100 µL of sterile 10 mM Tris and stored DNA frozen at -20°C.

Total DNA extraction from SW

DNA was extracted from 0.25 g of faeces using the MoBio PowerSoil® DNA Isolation Kit. For SW, 25 mL was filtered as described above, and DNA was extracted from the filter using the PowerWater® DNA Isolation Kit. DNA was extracted from the 10 g soil sample using the MoBio PowerMax® Soil DNA Isolation Kit. All extraction and filtering processes were undertaken in accordance with the manufacturers' instructions.

DNA test for 'human' faecal markers *Enterococcus faecium* and *Bacteroides thetaiotamicron*

A 680 bp fragment of the *E. faecium esp* gene and 542 bp fragment of the *B. thetaiotamicron* 16SrRNA gene were amplified by the polymerase chain reaction (PCR) using the MyTaq® PCR mix (Bioline, USA) in 50 µL volumes (Neave *et al*. 2014).

Statistical Comparisons

The Fisher Exact Probability Test was used in pair-wise comparisons between sites to determine differences in occurrence of the bacteria in faeces, soil and skin wash.

Results

Enterococci colonies grew in most faecal and soil plates regardless of location, but only grew in two skin wash samples, specifically from the suburban location (Fig. 1). The *E. faecium* test, using the plated enterococci DNA as template, was negative for all skin wash and soil samples. However, the *E. faecium* test was positive for some faecal samples, but only from the sewage-impacted site (Fig. 1), with the occurrence being significantly higher ($p = 0.04$) in this site compared to the suburban site.

The human faecal marker *B. thetaiotamicron* was detected in faecal DNA samples from all locations, but not in skin wash or soil samples (Fig. 1). The occurrence of *B. thetaiotamicron* was not significantly different among the three sites, including the reference site (p–values ranging between 0.07 – 0.56).

Discussion

The results of this study identified that the standard genus level test, using enterococci-specific media, detected enterococci in amphibian faeces. Sammon *et al.* (2009 and 2010) also reported that frog faeces were positive for *E. coli*, the other common faecal indicator bacteria. Our results indicate that there is reason to doubt the reliability of using either enterococci or *E. coli* as tests for mammalian faecal indicator bacteria. We also tested for *B. thetaiotaomicron* because it is considered to be an effective indicator of human faeces (Carson et al. 2005) and found that frog faecal DNA is positive for this bacteria. Given that both of the 'human' faecal indicator tests (*E. faecium* and *B. thetaiotamicron*) were positive in tree frog faeces, this adds to the growing evidence that currently used indicator bacteria are not as specific as initially believed (Layton *et al*. 2009; Radimersky *et al*. 2010). Although we cannot definitively state that the small sample of reference frogs had never been exposed to human sewage, it seems unlikely because they were found using natural tree hollows several kilometers from any buildings. Therefore, when questions specifically relate to human faecal contamination, results from currently used markers must be interpreted with caution.

However, these bacteria are nevertheless useful indicators of faeces, and for drinking water this would be a concern even if derived from an animal host because these bacteria occur in human faeces and may pose a potential public health risk. Our results show that frogs congregating in water tanks and defecating into the water could contaminate that water with these bacteria – and presumably other bacteria such as member of the *Firmicutes* commonly found in human faeces (Walker *et al*. 2011). Where there is no evidence of frog defecation, it is considered that these frogs do not pose the same type of health risk because there was no evidence that they can 'vector' these bacteria on their skin.

Importantly, *B. thetaiotamicron* was detected in frog faeces regardless of the presence of a source such as sewage effluent. These results indicate that frogs become hosts to faecal bacteria (that are commonly thought to be human-specific) whether they live in close contact with humans (i.e. suburban frogs) or in the natural environment. In contrast, *E. faecium* was only detected in frog faeces at the sewage-impacted site. This suggests the possibility that frogs become hosts to *E. faecium* only when they live in suburban areas contaminated with sewage effluent. However, a larger sample would need to be tested to confirm that pattern.

With respect to our original aims, we determined that frogs can host bacteria typically associated with human faeces, and although Enterococci colonies were present in both faecal and skin wash samples, the more specific markers (*E. faecium* and *B. thetaiotamicron*) were only found in faeces. With respect to our aim related to the source of the bacterial markers, faecal *B. thetaiotamicron* was detected from populations of frogs from both natural and urban sites, but *E. faecium* was only associated with the sewage impacted urban site. Both *E. faecium* and *B. thetaiotamicron* have been isolated from faeces collected from humans with symptoms of gastroenteritis (Ke *et al*. 1999; Kong *et al*. 2002; Teng *et al*. 2004). While more research would need to be done in this tropical context, the potential exists for people drinking water that contains frog faeces, to ingest bacteria that could replicate in their gut and cause illness. Moreover, other workers have shown that frogs contained *E. faecium* with antibiotic resistance conferred by a transposable element or 'jumping gene'. If these bacteria can be shed in drinking water, there is potential for their dissemination and uptake by humans (Rana *et al*. 2011).

Our recommendation therefore is that water tanks used to store drinking water should be properly constructed, sealed and maintained to guard against frogs. Our results are likely to be included in an update of the Commonwealth

of Australia Department of Health *Guidance on use of rainwater tanks* (2011), particularly the sections on microbiology testing and faecal contamination from small animals, with a specific recommendation about frogs.

Conclusions

(1) The human faecal indicator bacteria tested in this study were found in association with tree frog faeces, casting doubt on the validity of them being considered human or mammalian specific.

(2) *Bacteroides thetaiotamicron* was found in frogs faeces from all sites, including the natural site where the frogs lived in trees rather than being associated with human structures.

(3) *Enterococcus faecium* was only found in frog faeces from the suburban site near a creek that sometimes receives sewage effluent.

(4) Although we have not established a link between tree frogs and human health issues, these results suggest that it would be prudent to ensure drinking water tanks are constructed and sealed in such a manner to exclude entry by frogs.

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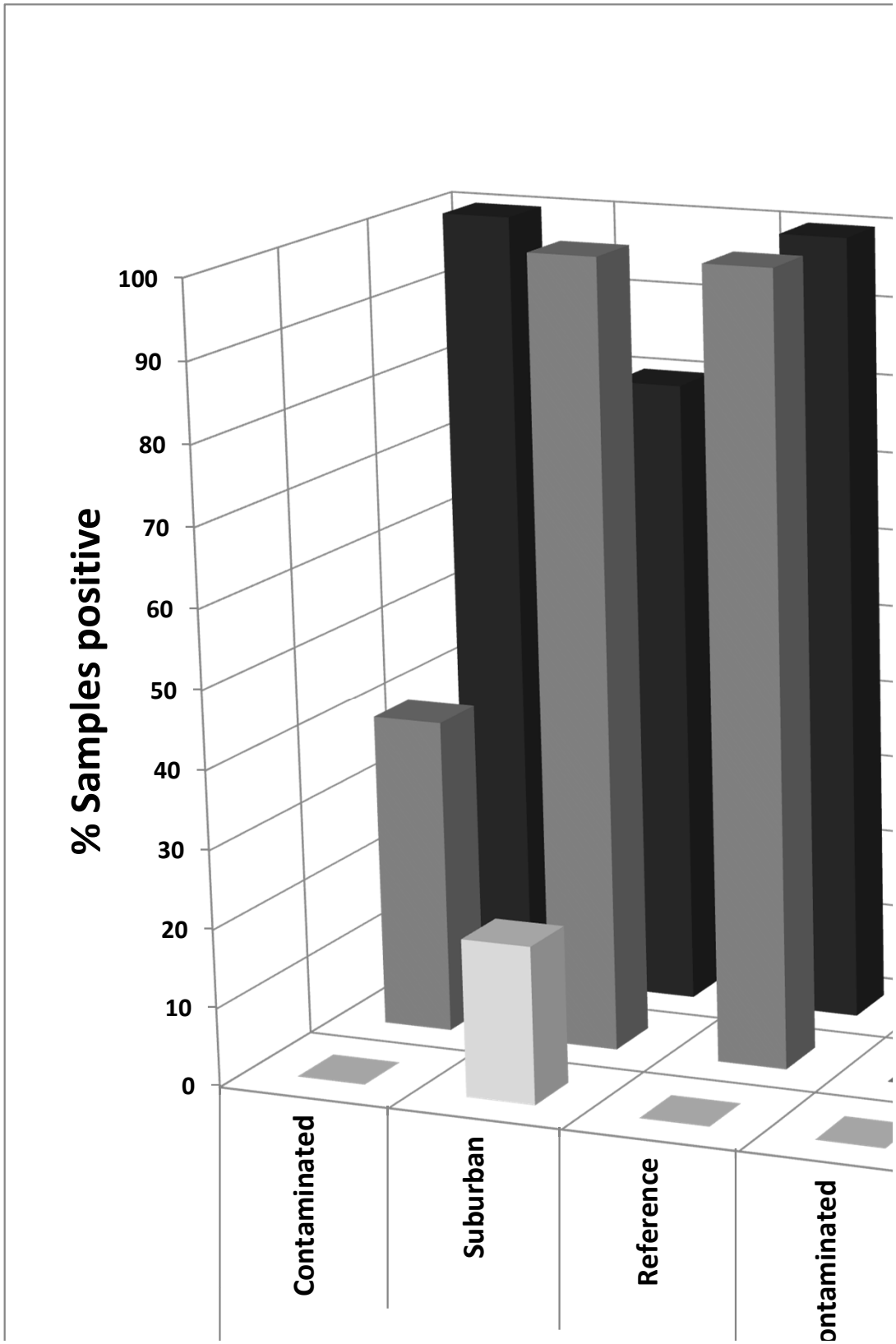
Fig. 1. The percentage of samples that were positive for Enterococci colonies, *E. faecium*, or *B. thetaiotamicron* from tree frog faeces, skin wash, and soil samples collected at three sites i.e. a natural reference, suburban, and contaminated (sewage impacted).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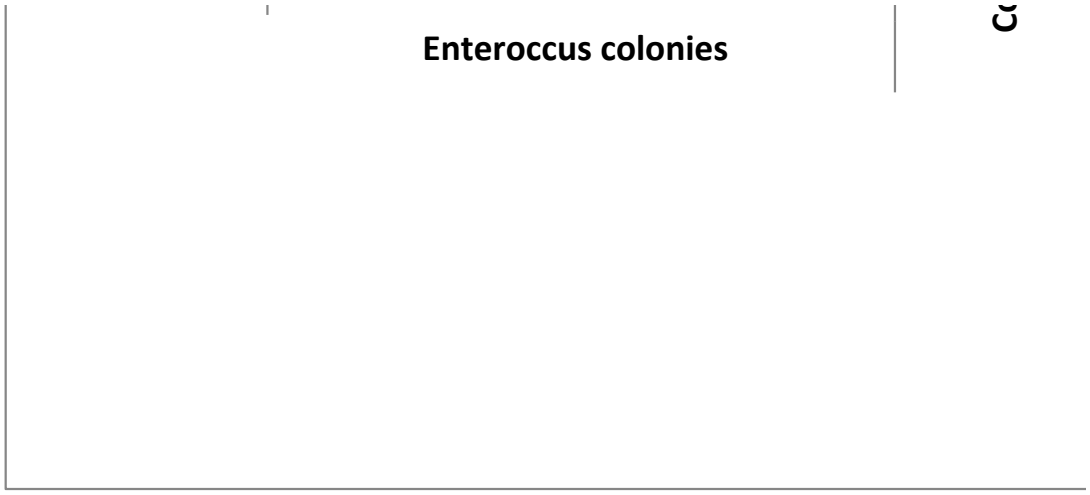

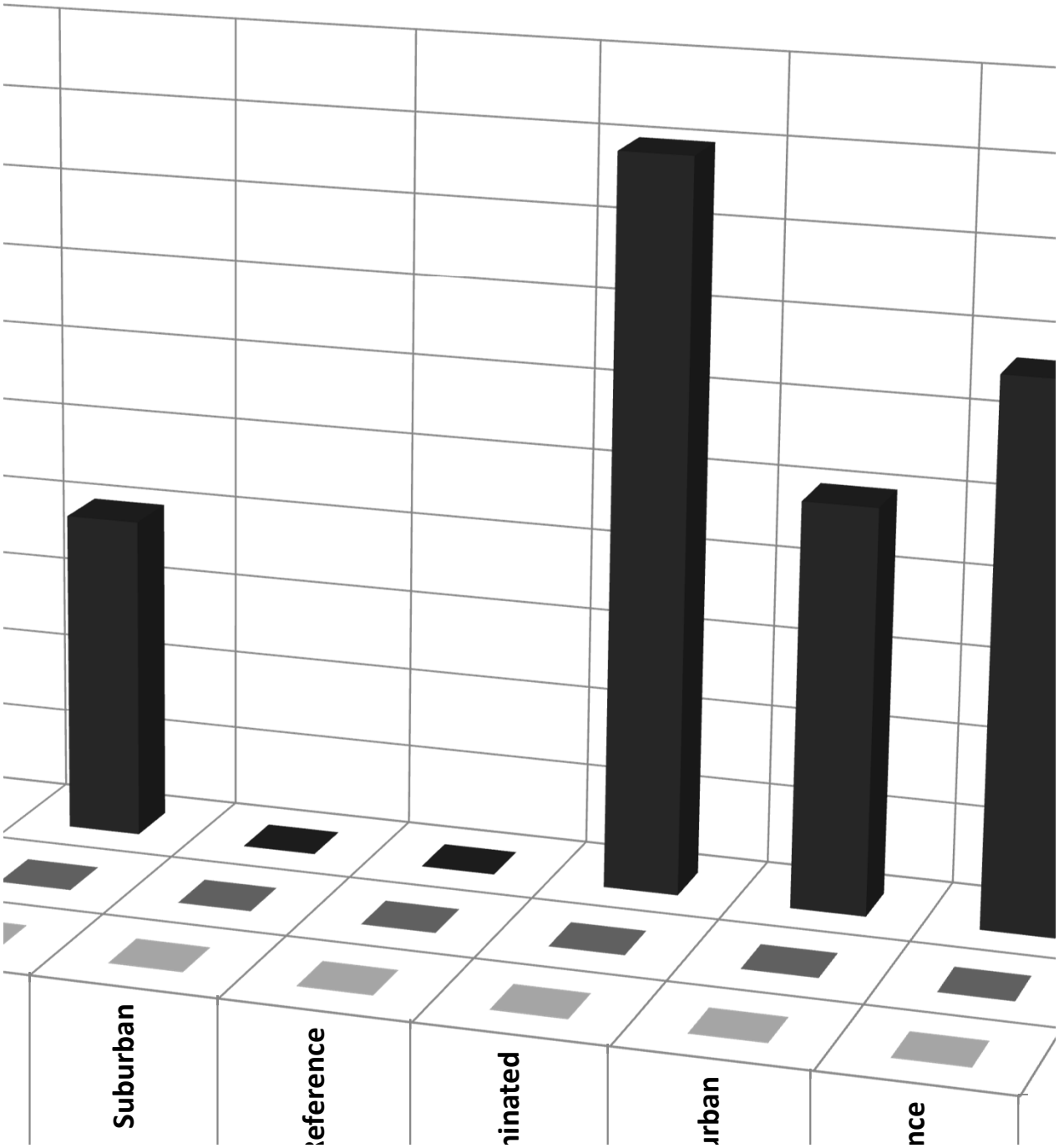
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