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8 **An assessment of drinking water contamination with *Helicobacter pylori* in Lima, Peru**

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27 **Abstract**

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Background: *Helicobacter pylori* is a gut bacterium that is the primary cause of gastric cancer. *H. pylori* infection has been consistently associated with lack of access to sanitation and clean drinking water. In this study, we conducted time-series sampling of drinking water in Lima, Peru to examine trends of *H. pylori* contamination and other water characteristics.

Materials and Methods: Drinking water samples were collected from a single faucet in Lima's Lince district five days per week from June 2015-May 2016, and pH, temperature, free available chlorine, and conductivity were measured. Quantities of *H. pylori* in all water samples were measured using quantitative polymerase chain reaction (qPCR). Relationships between the presence/absence and quantity of *H. pylori* and water characteristics in the 2015-2016 period were examined using regression methods accounting for the time series design.

Results: 49/241 (20.3%) of drinking water samples were contaminated with *H. pylori*. Statistical analyses identified no associations between sampling date and the likelihood of contamination with *H. pylori*. Statistically significant relationships were found between lower temperatures and a lower likelihood of the presence of *H. pylori* ($p<0.05$), as well as between higher pH and higher quantities of *H. pylori* ($p<0.05$).

Conclusions: This study has provided evidence of the presence of H. pylori DNA in the drinking water of a single drinking water faucet in the Lince district of Lima. However, no seasonal trends were observed. Further studies are needed to determine the presence of *H. pylori* in other drinking water sources in other districts in Lima, as well as to determine the viability of *H. pylori* in these water sources. Such studies would potentially allow for better understanding and estimates of the risk of infection due to exposure to *H. pylori* in drinking water.

## Introduction

*Helicobacter pylori* (*H. pylori*) is a stomach bacterium that, while asymptomatic in most people, can cause a cascade of gastric pathology leading to the development of gastric adenocarcinoma [1,2]. For this reason, it is categorized as a class 1 carcinogen [1,3]. *H. pylori* infection is hypothesized to be transmitted directly through fecal-oral, oral-oral, or gastro-oral routes, or indirectly through reservoirs, including food and water.[4–6] Although the global prevalence of *H. pylori* infection is decreasing, it is more prevalent in low-income nations as compared with high-income nations, with estimates in adults as low as 11% in Belgium and up to 93.6% in Nigeria.[7] Lima, Peru, where the first association between water source and *H. pylori*

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58 infection was discovered, has an estimated prevalence of 45.5%.[8] In 1991, Klein et al. found
59 higher odds of *H. pylori* infection among study participants in Lima with municipal drinking
60 water compared to those using private wells [4]. Since that time, lack of access to clean drinking
61 water and proper sanitation has been identified in epidemiological studies as a risk factor for *H.*
62 *pylori* infection [5,6,9–15].

63 *H. pylori* rapidly changes morphology from a spiral bacillus to a coccoid form in water,
64 entering a viable-but-not-culturable (VBNC) state that makes it challenging to culture and
65 renders cultivation techniques an inadequate stand-alone way of detecting *H. pylori* in water [16–
66 18]. Recently, however, five independent studies have isolated and cultured *H. pylori* in
67 wastewater and drinking water [19–23]. In addition, *H. pylori* has been reliably detected in
68 recreational and drinking water using molecular biology techniques such as PCR and fluorescent
69 in-situ hybridization [4,16,17,24–26], and has been shown to survive in water distribution systems,
70 likely through protection from biofilms [27,28]. Further, *H. pylori* may be able to better survive in
71 the presence of certain freshwater amoebae [29] and marine zooplankton [30], suggesting another
72 route of survival in water. While the VBNC form of *H. pylori* has been shown to be infectious in
73 mice via gavage [31,32] (but not in drinking water)[33], and viable, culturable *H. pylori* is infectious
74 through the route of drinking water in mice [34].

75 While it is plausible that water contaminated by *H. pylori* is a route for the transmission
76 of *H. pylori* infection, the quantities of *H. pylori* reported to be in drinking water and thus the
77 risk of infection from such sources remain poorly characterized. Existing studies have measured
78 the quantities of *H. pylori* in wastewater [25], surface water [35], and recreational water [36], and
79 several studies have measured the presence/absence of *H. pylori* in drinking water using
80 PCR [4,21,35] (50%, 28.6%, and 4% positive, respectively). To our knowledge, only two studies
81 have quantitatively measured *H. pylori* in municipal drinking water: one by our group in Lima,
82 Peru [26], and the other in Spain [39]. Both studies had limited sample sizes (n=87 and n=24,
83 respectively), and were conducted in a variety of locations. In Peru, the highest quantity of *H.*
84 *pylori* found in drinking water was 1.6E6 genome copies/L [26], which was remarkably similar to
85 Spain, where the highest quantity was reported as it was 1.59E3 genome copies/mL - equivalent
86 to 1.59E6 genome copies/L[39].

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To follow up our initial work in Lima, we performed two small studies. The first of these conducted in 2013 examined five water samples collected from wells used to supplement the drinking water downstream of the municipal treatment plant. These wells were located in the districts of Surco, El Agustino, Puente Piedra, and Comas. Second, 17 drinking water samples were collected between June 19$^{th}$ and July 18$^{th}$ of 2014 from a single sink in the Lince district in Lima. Samples were collected once to twice per week, except during the week of June 30$^{th}$-July 4$^{th}$, when samples were collected twice per day from Monday to Friday. 3/5 samples collected from wells and 9/17 drinking water samples collected in the summer of 2014 showed contamination with *H. pylori*, with well water samples containing up to 2.3E3 genome copies/L of *H. pylori* and drinking water samples containing up to 1.95E4 genome copies/L of *H. pylori* (Xi lab, unpublished data).

To follow up our studies and better characterize the quantities and variation of *H. pylori* in drinking water over time, we then conducted a quantitative assessment of *H. pylori* in water in the Lince district in Lima, Peru.

## Materials and Methods

### *Water sample collection*

241 total drinking water samples were collected once per day, five days per week from a single sink within a government building in the Lince district in Lima from June 2015 through the end of May 2016. Approximately 20 people utilize this sink regularly. Sterile bottles with sodium thiosulfate were prepared prior to sampling. 1L aliquots of drinking water were collected from the faucet after allowing the water to run for at least one minute, and then concentrated by vacuum filtration onto 0.22μM filter membranes [26]. Water quality parameters including pH, temperature, and conductivity were monitored, and free available chlorine was measured using N,N Diethyl-1,4Phenylenediamine Sulfate (DPD) among samples collected from June 2015 to May 2016. Samples were handled per the US Geological Survey guidelines [40]. All membranes were stored at -80°C until processing and analysis at the University of Michigan.

### *DNA extraction from membranes*

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The following phenol chloroform protocol for DNA extraction was adapted from Holinger et al. 2014 [41]. Each 0.22 μM filter (EMD Millipore, Ontario, Canada) was cut into >20 pieces using sterilized scissors, and placed into 2mL tubes containing ~0.5 g of 0.1 mm silica/zirconium beads (Biospec Products, OK, USA), 500 μL of phenol/chloroform/isoamyl (25:24:1) and 500 μL of lysis buffer (75mM NaCl, 75mM TRIS pH 8.0, 7.5mM EDTA, 2.85% SDS). Samples were mechanically bead beaten for 2.5 minutes at high speed to separate cells from the membrane and lyse the cells. To separate phases, tubes were centrifuged for 7 minutes at 16,000x g. Following centrifugation, ~450 μL of aqueous phase was transferred to a new 1.5 mL tube. 10 μL of glycogen (10 mg/mL), 200 μL of 7.5M-ammonium acetate and 650 μL of isopropanol were added to precipitate the DNA. The samples were then centrifuged for 25 minutes at 16,000x g to pellet the DNA. Afterwards, the supernatant was removed, and pellets were washed with 1 mL of cold 70% ethanol. The samples were then inverted 15-30 times and centrifuged for 10 minutes at 16,000x g. After removal of ethanol, the pellets were dried at 35°C for 1-2 hours using a vacuum spinner. DNA Pellets were suspended in 40 μL of 10mM TRIS with 1mM EDTA, pH 8.0. Following DNA extraction, samples were purified by washing with 1mL of 4°C 70% ethanol and 10 μL of 3M-sodium acetate. After inverting the sample tubes 15-30 times, they were centrifuged for 10 min at 16,000x g. Ethanol was removed, and pellets were dried at 35°C for 1-2 hours using a vacuum spinner. Pellets were again suspended in 40 μL of 10mM TRIS and 1mM EDTA, pH 8.0.

*qPCR (quantitative polymerase chain reaction)*

qPCR was performed on extracted DNA using a highly sensitive, previously established method [42]. Briefly, the number of genome copies/L of *H. pylori* in drinking water was quantified using a reaction mixture containing 10 μL 2×SYBR GREEN PCR Master Mix (Applied Biosystems, Grand Island, NY, USA), 0.3 μL of each 20 μM primers HpA-F (ACTTTCTCGCTAGCTGGATGGTA) and HpA-R (GCGAGCGTGGTGGCTTT), 8.9μL of sterile PCR water, and 0.5 μL of DNA template. Plates also included negative controls (no DNA added) and positive controls (*H. pylori* strain SS1 DNA), and a standard curve was constructed with 0.5E1 to 5E5 genome copies of *H. pylori* strain SS1 DNA. Given that each *H. pylori* genome has 1 copy of *hpaA*,[43] we assumed that one genome copy of *H. pylori* was equivalent to 1 genomic unit (GU). The lowest value (0.5E1 genome copies) was used to determine the limit

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146 of detection in calculating the quantity of *H. pylori* in each sample. qPCR was run under the
147 following conditions: 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 1
148 minute, followed by a melting curve analysis, ramping from 60°C to 95°. All drinking water
149 samples, standard curve samples, and controls were run in triplicate.

150

151 *Statistical analysis*

152 All statistical analyses were conducted in R Studio version 0.99.891. Descriptive statistics were
153 used to examine the distribution of *H. pylori* contamination, pH, temperature, conductivity, and
154 free available chlorine. Time-series plots were constructed to examine potential seasonal patterns
155 in water characteristics.

156 Due the large number of negative samples (~80%), two models were run to account for
157 zero-inflated data. The first was a logistic regression that modeled the presence/absence of *H.*
158 *pylori*, based on all samples. The second was a linear regression modeling the quantity of *H.*
159 *pylori* as an outcome, run on non-zero samples only. Put together, these regressions model the
160 presence or absence of *H. pylori* in the sample, and, conditional on being a positive sample, the
161 quantity present. Both models were adjusted for all water characteristic co-variates. Since the
162 data arise as a time series because sampling occurred from a single location over the course of
163 one year, two approaches were used to account for the possibility of autocorrelation in the
164 samples (i.e., non-independence from day-to-day). First, the prior day's presence/absence of *H.*
165 *pylori* contamination was included as a predictor in both models. Second, the autocorrelative
166 effects of date on the presence/absence of *H. pylori* was measured in the logistic regression by
167 incorporating a smooth function of date of sample using the R 'gam' package. The smooth
168 function adjusts for autocorrelation by modeling the potential long-term calendar trends in the
169 presence/absence of *H. pylori*. Smoothing functions were also used to investigate potential non-
170 linearity in the effect of the remaining water characteristics on the presence of *H. pylori.*

171 Model residuals were used to examine model fit and to identify potentially outlying
172 values. The influence of extreme values was examined by removing these values from the model
173 and examining the resulting robustness of the models. Due to the highly-skewed distribution of
174 residuals, the quantities of *H. pylori* were log-transformed in the linear regression model.

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175 **Results**

176 *H. pylori in drinking water*

177 Throughout the sampling period (June 2015 to May 2016) contamination of the drinking water
178 with H. pylori was observed, with 23% (49/241) of samples being positive for H. pylori. Each
179 month H. pylori contamination was detected on at least one day, with the longest stretch of time
180 without a positive sample being 25 days (Fig 1 A). In this figure, the limit of detection is
181 reported as 400 genome copies/L, due to back-calculation from the total elution volume and
182 quantity of sample used per reaction [(5 genome copies/well) * (well/0.5 µL sample) * (40 µL of
183 total DNA/L drinking water sample)].

184 **Figure 1.**

185 *Water characteristics*

186 There were some missing measurements in the water characteristics data due to lack of reagent
187 or instrumentation on that sampling day (Table 1, Figure 1B, 1C, 1D, 1E). pH was measured in
188 238/241 samples, conductivity in 232/241, free available chlorine in 209/241, and temperature in
189 240/241. The World Health Organization (WHO) recommends that the free chlorine residual
190 available in drinking water should be between 0.2 and 0.5mg/L [44]. 169/209 samples were at or
191 below 0.5 mg/L, 20/209 samples were above 0.5mg/L, and 17/209 samples were below the
192 minimum recommended residual of 0.2mg/L of free chlorine (Table 1). The WHO recommends
193 that the pH of drinking water should be above 6.5 and below 8.5 to avoid corrosion 5 [45]. 96/238
194 samples were below the minimum recommended guideline of 6.5. Temperatures ranged from
195 19.7-27.7°C, and conductivity ranged from 326-616 µS/cm.

196

197 **Table 1. Water characteristics from June 2015-May 2016 sampling campaign**

| | **N** | **Range** | **Median** | **Mean** | **Notes** |
|---|---|---|---|---|---|
| *H. pylori* genome copies/L | 241 | 0-2.56E3 | 0 | 60.68 | 49/241 samples positive, 12 above the detectable limit |
| pH | 238 | 5.16-8.43 | 6.68 | 6.54 | 96 samples were below the EPA recommendation of 6.5 (too acidic) |

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| Temperature in Celsius | 240 | 19.7-27.7 | 23.3 | 23.6 | Fairly high temperatures, good conditions for bacterial growth |
|---|---|---|---|---|---|
| Conductivity in µS/cm | 232 | 326-616 | 512 | 504.8 | |
| Free chlorine residual (FAC) in mg/L | 209 | 0.01-0.78 | 0.37 | 0.3658 | 17/209 samples below WHO recommendation of 0.2-0.5 mg/L of FAC |

Table 1. Summary of water characteristics from sampling campaign in Lima from June 2015 through May 2016. Note: Not all water characteristics were measured in all samples due to lack of reagent or instrumentation on that sampling day.

*Associations between water characteristics and H. pylori*

We found a significant negative association between temperature and presence of *H. pylori*, regardless of the method of analysis. Accounting for date using a smoothing function and all other co-variates, we found that the log odds of the presence of *H. pylori* was 37% lower (ß=-0.46, SE=0.18, $p<0.05$) per degree higher temperature (Table 2, 1a). When accounting for autocorrelation with the previous date (Table 2, 1b), the log odds of the presence of *H. pylori* was 21% lower per degree higher temperature (ß=-0.24, SE=0.13, $p<0.1$) (Table 2, 1b). Temperature remained statistically significant in the smoothed model, even when the most influential point was removed ($p<0.05$). Although the approach to adjusting for auto correlation (smoothing vs. adjusting for prior day) changes the direction of the association with pH, pH was not significantly related to presence/absence of *H. pylori* in either model.

In the log-transformed linear regression models incorporating only positive samples, we found a significant positive association between pH and the quantity of *H. pylori* and a marginally significant negative association between conductivity and quantity of *H. pylori.* The quantity of *H. pylori* was 139% higher for each unit higher in pH, and 0.62% lower per µS/cm higher of conductivity (ß=-0.0027, SE=0.0015, $p<0.1$). After removing the most influential data point, the quantity of *H. pylori* was 95% higher for each unit higher in pH (ß=0.29, SE=0.14, $p<0.1$, Table 2, 2b).

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220 We did not find a statistically significant association between calendar time (i.e., long-
221 term seasonal trends) and the presence of *H. pylori* (figure not shown). Similarly, we did not find
222 association between the presence of *H. pylori* in a given sample and presence of *H. pylori* in the
223 prior day's sample.

224 **Table 2:** Statistical models examining the relationship of pH, free available chlorine residual,
225 conductivity, temperature, and the presence/absence or quantity of *H. pylori*.

| Variables | Model 1a: Logistic Regression for presence/absence of *H. pylori* with smoothing (ß, SE). | Model 1b: Logistic Regression for presence/absence of *H. pylori* - autocorrelation adjustment (ß, SE). | Model 2a: Log-transformed Simple Linear Regression for quantities of *H. pylori* (ß, SE). | Model 2b: Log-transformed Simple linear Regression for quantities of *H. pylori* (ß, SE) with influential outlier removed . |
|---|---|---|---|---|
| Intercept | 9.73 (5.42) | 5.41 (5.12) | 0.29 (2.25) | 1.54 (2.31) |
| Prior day presence/absence of *H. pylori* | N/A | 0.27 (0.43) | 0.03 (0.18) | 0.034 (0.18) |
| Cl2 residual | -0.33 (1.6) | -0.99 (1.58) | 0.56 (0.67) | 0.28 (0.67) |
| pH | 0.23 (0.33) | -0.04 (0.31) | 0.38 (0.14)** | 0.29 (0.14)* |
| Conductivity | -0.003 (0.004) | -0.001 (0.004) | -0.0027 (0.0015)* | -0.002 (0.0015) |
| Temperature | -0.46 (0.18) ** | -0.24 (0.13)* | 0.025 (0.57) | -0.017 (0.06) |

226 Table 2. *=p<0.1, **=p<0.05.

227 **Discussion**

228 To our knowledge, this is the longest time-series sampling study of drinking water contamination
229 with *H. pylori* in Lima, Peru. Based on our sampling, it appears that drinking water in Lima is
230 contaminated with *H. pylori* 20.3% of the time (49/241 positive samples), in both the drinking
231 water collected in Lince and in the wells used to supplement the treated drinking water supply.
232 Based on the null associations found between presence/absence of *H. pylori* and the prior day's

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sample, there is no strong autocorrelation to indicate any seasonal trends, suggesting that contamination occurs randomly over time in this location.

While we found statistically significant relationships between temperature and the presence/absence of *H. pylori* and between pH, conductivity, and the quantity of *H. pylori*, inference from our data is somewhat difficult. In a previous laboratory study, the optimal pH for *H. pylori* survival in water was found to be 5.8-6.9, but that does not account for other relevant factors, such as co-exposure with chlorine [46]. Multiple studies have shown that *H. pylori* can survive longer at lower temperatures in both well water and river water. [16,47] However, since all cells went into the VBNC state, the authors could not comment on persistence or reproduction at these temperatures. Although our results are in line with other literature, more research is needed to tease out whether *H. pylori* dies more quickly at higher temperature, or simply moves into a VBNC state more rapidly. The lack of an explanatory mechanism for how pH might be positively associated with the quantity of *H. pylori* makes it uncertain whether these relationships are meaningful. Given this, it seems likely that other, unaccounted-for biotic and abiotic factors might be important in relation to the presence or quantity of *H. pylori* contamination, such as the frequency of infusions of well-water from contaminated wells, contamination from leaks in the distribution system, and the stochastic shedding of cells from biofilms in the pipes.

Despite this, our current study shows that there is contamination of drinking water with *H. pylori* in Lima, Peru. Since 1996, there have been mixed reports of the presence of *H. pylori* in drinking water. Investigations in Peru [4,26], Sweden [48], Pakistan [37], Iraq [19], Iran [22,49], Costa Rica [23], and Spain [39] have shown contamination of drinking water with *H. pylori* using PCR, culture, and microscopy techniques such as fluorescent in-situ hybridization. In contrast, studies in Bangladesh [42] and Japan [38] failed to detect *H. pylori* in treated drinking water, though the study in Japan and a further study in Scandinavia detected *H. pylori* via PCR in untreated well-water used as drinking water [10,38]. The results from our study are consistent with those that reported contamination, though the scope of our sampling, in terms of length of time and number of samples, was wider than any previously reported study. The quantities of *H. pylori* we found were also substantially lower than those reported in Spain and Peru, with our highest value being 2.5E3 genome copies/L, compared to 1.59 and 1.6E6 genome copies/L, respectively.[26,39]

*Limitations*

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263 Since we used a DNA-based method of detection, we could not determine between viable
264 and non-viable *H. pylori* cells in drinking water, so we are unable to infer whether the DNA
265 amplified was from culturable, viable but non-culturable, or non-viable *H. pylori* cells. Thus,
266 examining our results in a risk assessment format would be problematic, as we cannot determine
267 the relative proportions of each form, and the relative infectiousness of the VBNC compared to
268 the culturable form is not well characterized. However, Sen et al. (2011) found that *H. pylori*
269 DNA cannot be amplified after exposure to chlorine in tap water for 2-3 days; a period of time
270 that it typically takes for water to go from the treatment plant to a household [50]. While this may
271 suggest that the DNA detected in our and other studies in chlorinated drinking water may have
272 come from VBNC or viable, culturable *H. pylori* cells, it is in no way conclusive.

273 Further, it is uncertain whether water in the Lince district is representative of water
274 elsewhere in Lima. Based on our previous cross-sectional sampling through the city of Lima,
275 there appeared to be widespread of contamination that this did not appear to be linked to a
276 specific district [26]. Given that there are leaks in the distribution system and the large amount of
277 unaccounted-for water [51], it is possible that some areas of Lima might have more contamination
278 than others. Further studies are needed throughout the city to examine whether such
279 contamination is systemic.

280 *Public Health Implications*

281 Other studies in the literature provide more of a snapshot of water contamination with *H. pylori*,
282 collecting samples either once or a handful of times from multiple locations [10,26,39]. By collecting
283 water from a single location over a year, we better characterized the annual body burden of *H.*
284 *pylori* from drinking water in the Lince district in Lima, which can be used to more accurately
285 assess risk of infection from this exposure route. However, this relationship may be modified by
286 downstream water treatment techniques, such as boiling and bleach disinfection. Indeed,
287 according to a survey by the World Bank's Water and Sanitation Program, 89% of people treat
288 tap water (primarily through boiling) before they drink it.[52]

289 The biggest limitation of our and other quantitative surveys of *H. pylori* contamination of
290 drinking water is that they could not distinguish between VBNC, viable culturable, and non-
291 viable *H. pylori* cells. Thus, although several samples in our and other studies [26,39] had quantities
292 of *H. pylori* found to be infectious in either mice, humans, or monkeys [34,53,54], it is unclear

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whether the sampled water poses the same infectious risk found in dosing trials since these trials used the viable, culturable state of *H. pylori*. In the only quantitative microbial risk assessment performed thus far for *H. pylori* in drinking water, Ryan et al. (2014) recommended that the maximum contaminant level goal for *H. pylori* be set at $<1$ organism/L in drinking water based on the downstream risk of infection and gastric cancer [55]. That study used quantities of *H. pylori* found in surface and recreational water [35,36], which, when accounting for the efficiency of municipal water treatment in the USA, were substantially lower than those found in our study. Further, the contamination of treated water from La Atarjea [4,26] and the consistent contamination of well-water used to supplement the treated drinking water (Xi et al., unpublished data) highlights the need for point of use water treatment options and long term investment in water treatment infrastructure to provide safe, potable water to the populace of Lima.

## Conclusions

Over a one-year sampling period, we detected *H. pylori* in 20.3% of drinking water samples from Lima, Peru using qPCR, which suggests that there is continued contamination of the water supply in the Lince district. We found no significant relationship between sampling date and likelihood of *H. pylori* contamination, but found that increased temperature was associated with a lower likelihood of presence of *H. pylori* and that increased pH was associated with a higher quantity of *H. pylori*. Further studies are required to examine whether this is true in other districts in Lima. Future studies should aim to identify potential sources for contamination and better characterize the risk of *H. pylori* in drinking water, as well as examine the effectiveness of downstream drinking water treatments, such as boiling. Given that gastric cancer is the most common cause of cancer mortality in Peru, these findings highlight the need for effective point-of-use household water treatment in the short term, and long-term investment in infrastructure to provide high quality drinking water for the citizens of Lima, Peru.

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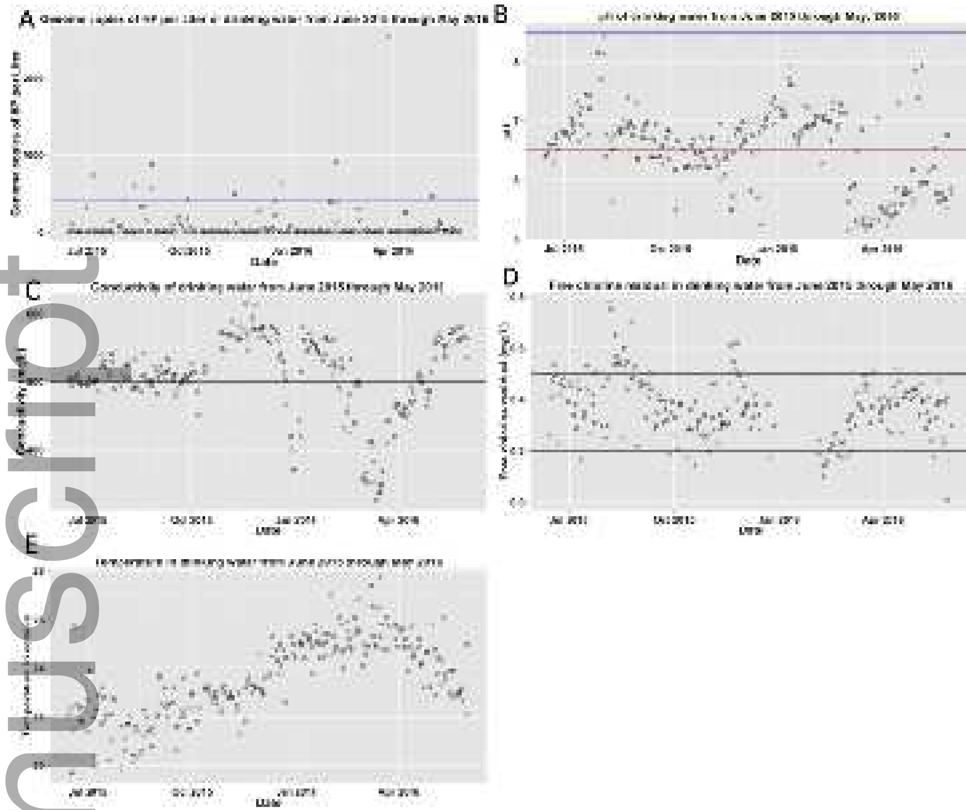


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