TITLE An evaluation of urine patch simulation methods for nitrous oxide emission measurement

AUTHORS FORRESTAL, P., KROL, D., LANIGAN, G., JAHANGIR, M., & RICHARDS, K.

This article is provided by the author(s) and Teagasc T-Stór in accordance with publisher policies.

Please cite the published version.

The correct citation is available in the T-Stór record for this article.

NOTICE: This is the author’s version of a work that was accepted for publication in Journal of Agricultural Science © Cambridge University Press 2016 the Version of Record can be accessed at doi: http://dx.doi.org/10.1017/S0021859616000939

This item is made available to you under the Creative Commons Attribution-Non commercial-No Derivatives 3.0 License.
Title: An evaluation of urine patch simulation methods for nitrous oxide emission measurement

Summary

Global N$_2$O-N inventory estimates for pasture systems are refined based on measurements of N$_2$O-N loss from simulated urine patches using a variety of methods but frequently using a uniform wetted area (UWA), often smaller than a bovine urine patch. However, natural patches follow non-uniform infiltration patterns expanding naturally from a point of deposit with a non-wetted zone of influence. Using 2 L urine the UWA method was compared, using a 0.156 m$^2$ collar, with a naturally expanding effective area (NEEA) method, using a 0.462 m$^2$ collar under high (HL) and low (LL) N$_2$O loss conditions. The method chosen affects urine N loading to the soil. Under HL the UWA method induced a N$_2$O-N loss of 280.6 mg/patch, significantly less than the 434.8 mg/patch loss for the NEEA method, for the same simulated urination. Under LL there was no method effect. Efforts should be made to employ patch simulation methods which mimic natural deposits and can be achieved, at least in part, by:

a) use of a urine volume and N content similar to that of the animal of interest.
b) allow natural infiltration of the chosen urine volume to permit tapering toward the edges.
c) measure from the zone of influence in addition to the wetted area i.e. the patch effective area.

Keywords: nitrous oxide, urine, patch, denitrification, pasture range paddock
Introduction

Nitrogen (N) inputs to agricultural soils contribute to the production of the greenhouse gas, nitrous oxide (N₂O) and animal production accounts for an estimated 1.5 Tg N₂O-N/yr (Oenema, et al. 2005). In pasture systems, urination by grazing animals causes a mosaic of discrete patches of highly concentrated N loading to soil. Approximately 41% of N₂O-N emissions from animal production are attributable to urine and dung deposition by grazing animals (Oenema, et al. 2005). An increasing number of studies have focused on a) quantifying N₂O-N emissions from urine, and b) assessing urine N₂O-N emission mitigation strategies in pasture systems. These studies typically use simulated urine patches (Table 1). Natural urine patches are intrinsically heterogeneous in their within-patch N loading and size. Selbie et al. (2014) summarized the drivers of this variability as urine volume, wind, slope, antecedent soil moisture and soil physical properties. Cattle urine patches were observed to range from 0.16 to 0.49 m² by Williams & Haynes (1994), to have a mean patch area of 0.353 m² (Saarijarvi & Virkajarvi, 2009) and to expand naturally over time (Williams & Haynes, 1994). Dairy cow urine patches (4 year mean 0.37 m²) have also been measured using the zone of grass response as a proxy for the urine wetting front (Moir et al. 2010). Saarijarvi & Virkajarvi (2009) reported that the non-wetted zone of influence extended up to 150 mm from the wetted patch edge. The total area is termed the “effective area” of a urine patch (Selbie et al. 2014). It follows that effective area of the patch be expected to delineate the zone of increased N₂O loss potential associated with a urine deposition. There is considerable variability in methods used to simulate urine patches for N₂O loss estimation (Table 1). The two most common methods are to uniformly apply urine to either a) a defined area larger than the footprint of the N₂O measurement collar and subsequently install the collar or b) install the collar prior to application to constrain urine (Table 1). These methods, though practical, do not perfectly simulate a naturally occurring urine patch for a
number of reasons. First, they create a uniformly wetted area. Secondly, when constrained by a collar, urine infiltration along the horizontal plain in the surface soil, the most active zone of denitrification (Luo et al. 1998), is restricted. Thirdly, the constraint interferes with the pattern of urine interaction with soil. Fourthly, there are discrepancies between the average footprints of naturally deposited urine patches and the collars used to simulate them (Table 1). In recent work, Rochette et al. (2014) took an alternative approach by simulating a urine patch with a wetted area which was 33% of the N₂O measurement collar area thus ensuring the zone of influence was accounted for.

The objective of this work was to summarise patch simulation approaches in the literature and to evaluate the hypothesis that the N₂O loss induced by a simulated dairy cow urination would be affected by patch simulation and measurement approach. The typical “uniform wetted area” (UWA) method which artificially limits horizontal movement of urine, is compared to a “natural expanding effective area” (NEEA) method using a collar large enough to allow natural infiltration of urine.

Materials and methods

Site description, experimental design, and treatments

Field experiments were conducted under two conditions i) “high” N₂O loss, which occurred at a moderately drained site in autumn (HL) and ii) “low” N₂O loss, which occurred at a freely draining site in spring (LL). This approach permitted comparison of the methods under contrasting loss conditions and was not designed to explore specific site or seasonal differences which are heavily influenced by specific soil and environmental factors following treatment application. The HL occurred on a moderately draining Cambisol (58% sand, 30% silt, 12% clay, 7.3% organic matter, 3.2% total C, 0.30% total N, pH 5.7 0-10 cm) in autumn 2013 at the Teagasc Johnstown Castle Research Centre, Co. Wexford, Ireland (52°18’N; 6°
occurred on a free-draining Cambisol (58% sand, 28% silt, 14% clay, 7.9% organic matter, 3% total C, 0.32% total N, pH 5.8 0-10 cm) in spring 2014 at the Teagasc Moorepark Research Centre, Co. Cork, Ireland (52°09’N; 8° 14’W). Both sites were in long-term grassland dominated by perennial ryegrass (*Lolium perenne* L.). No organic manures or fertilisers were applied and animals were excluded for a period of at least six months in advance of the experiments. Grass was cut to approximately 5 cm before the experiments and allowed to regrow to approximately 8 cm. Stainless steel N$_2$O measurement collars were inserted to 7 to 10 cm depth at least four days prior to treatment application. Soil volumetric moisture (0-10 cm) was measured using a Theta probe soil moisture sensor (Delta-T, Cambridge, U.K.) in the area surrounding the simulated urine patches. Soil bulk density (0-10 cm) was measured to calculate water filled pore space (WFPS) per Maljanen *et al.* (2007). Precipitation, air and soil temperature (0-10 cm) were measured at a nearby (<500 m) meteorological station.

The treatments were a) UWA, a patch simulated by uniformly applying 2 L of urine within 0.156 m$^2$ collars and b) NEEA which closely mimicked a natural urination by applying 2 L urine to a central point within collars of 0.462 m$^2$ and allowing urine migrate outward as it would naturally. Although the simulated patches originated from the same simulated urination (2 L) the UWA method resulted in a uniform volume loading of 12.8 L/m$^2$ and the NEEA a non-uniform urine loading with a mean of 4.33 L/m$^2$. The urine N loading differed on an area basis but not on a simulated urination basis or on a patch basis. This is an important point because it is the N$_2$O-N emission associated with a urination voided by an animal which represents the unit of interest. The control treatment to measure the soil background N$_2$O emission (control) used a 0.156 m$^2$ collar. Up-scaling N$_2$O emissions from a chamber scale to area scales is a common practice for presenting results, in a like manner the background emission for a 0.462 m$^2$ area was calculated by up-scaling emissions from 0.156
Treatments were applied on the morning of 14 October 2013 and 8 April 2014 for the HL and LL experiments, respectively. The experimental design was a randomised block design, with three treatments (UWA, NEEA and untreated control) present in each of the five replicate blocks. The experimental unit was the plot, which in all blocks contained one simulated urine patch per urine treatment dedicated to N\(_2\)O sampling. Additionally, in blocks 1, 3 and 5 each experimental unit contained an additional individual simulated urine patch (HL) or three additional simulated urine patches (LL) which were used solely for soil sampling and mineral N assessment. The dimension of these experimental units was 4 m by 2.5 m. For the experimental units containing one urine patch the plot size was 1.5 m by 2.5 m with the treatment located centrally in the plot.

Urine was collected from grazing lactating Holstein Friesian dairy cows less than a week prior to application, homogenised and refrigerated at 4°C until application. The urine N content was measured using an Aquakem 600 discreet analyser (Cabrera and Beare, 1993). The urine N content at application was 8.3 g/L and 5.3 g/L for the HL and LL experiments, respectively.

**Nitrous oxide sampling and analysis**

Unvented stainless steel covers (10 cm in height) were used to form a headspace. Chamber to collar sealing was via a neoprene gasket, compressed by a 6 kg weight. A 10 mL gas sample was taken through a rubber septum after 40 minutes (Becton Dickinson, UK) using a 10 mL polypropylene syringe (BD Plastipak, Becton Dickinson, UK) fitted with a hypodermic needle (BD Microlance 3, Becton Dickinson, UK) and was injected into pre-evacuated 7 mL screw-cap septum glass vials (Labco, UK). The N\(_2\)O sampling procedure of Chadwick *et al.* (2014) was followed. Eight samples of ambient air were collected at each sampling. Their mean N\(_2\)O concentration was set as a surrogate for N\(_2\)O concentration at time zero. The assumption of a linear increase in headspace N\(_2\)O accumulation (Chadwick *et al.*, 2014)
during the 40 minute enclosure period was verified on each sampling occasion by collecting five headspace samples per chamber from a random sub-set of urine treated chambers during a 60 minute enclosure period. Of the sub-set of chambers which had a flux, 87% were linear according to the criteria of Chadwick et al. (2014). At the end of the 60 minute enclosure period the mean nitrous oxide concentration inside chambers in the linear group was 3.5 ppm (standard deviation 3.96 ppm). For the quadratic group it was 2.62 ppm (standard deviation 1.93 ppm). The quadratic group was not dominated by any particular urine treatment. The methodology of Chadwick et al. (2014) has been used in the generation of emission factors e.g. Bell et al. (2016), Krol et al. (2016) and treatment inter-comparison e.g. Minet et al. (2016). Nitrous oxide concentrations were determined using a gas chromatograph (GC) (Varian CP 3800 GC, Varian, USA). Hourly N₂O emissions were calculated based on the rate of N₂O concentration change during the enclosure period. Flux calculations accounted for air temperature, atmospheric pressure, and the ratio of surface area to chamber volume. Sampling took place between 10 and 12 am and was used to calculate daily emissions (de Klein et al., 2003). Cumulative emissions were calculated by integrating the daily fluxes and linear interpolation between measurement points (de Klein & Harvey, 2012) over 66 and 70 days in the HL and LL experiments, respectively. In each experiment sampling was conducted on 20 occasions with the highest sampling intensity following treatment application (Fig. 3).

Soil sampling and analysis

Soil samples (0-10 cm) were collected were collected by sampling at 15 cm intervals across a horizontal cross-section of each patch to obtain a composite sample. In total there were 12 soil samplings in the HL and 7 in the LL experiment (Fig. 2). Samples were fresh sieved using a four mm sieve, sub-sample gravimetric moisture content and mineral N content was
measured. Samples were extracted with 2M KCl and mineral N in the extract was determined using an Aquakem 600 discrete analyser.

**Data presentation and statistical analysis**

The flux data is presented per simulated urine patch as has previously been done by Rochette *et al.* (2014). The effect of treatment and time after urine application on the dependent variables of N₂O, soil NO₃-N and NH₄-N were evaluated using the REPEATED statement of the PROC MIXED procedure of SAS 9.3 (© 2002-2010, SAS Institute Inc., Cary, NC, USA). The factors in the model were treatment, time of sampling and block with time of sampling as the repeated factor. The pooled standard error of the mean is presented in Figures. The treatment effect on the cumulative mass of N₂O-N loss during the measurement period was tested using the PROC GLMMIX procedure of SAS. This analysis included treatment, loss condition i.e. HL or LL and their interaction as fixed effects and block as a random effect.

**Results and discussion**

WFPS is an important driver of N₂O-N loss (Smith *et al*., 1998). Conditions were not favourable for N₂O loss under LL due to lower WFPS levels (45-55%). Under LL the urine treatments were not significantly different from the control (Table 2). Consequently, it is not surprising that patch simulation approach had no effect. In contrast, under the HL conditions precipitation occurred almost daily following urine application (Fig. 1a) and WFPS exceeded 65% for at least 40 days following urine application. Additionally, soil temperature at patch simulation, a time when N₂O-N losses are frequently greatest (Williams *et al*. 1999; Maljanen *et al*. 2007; Krol *et al*. 2015), was also three to five degrees Celsius higher. Smith *et al*. (1998) reported an exponential increase in N₂O production related to temperature. Under these conditions both urine treatments increased N₂O loss significantly compared with the
control ($P<0.001$). The NEEA which closely mimics a natural urine deposit induced a significantly greater loss compared with the UWA method ($P<0.01$). The UWA patch had a net relative emission of 64% compared to the NEEA method (Table 2). An important factor explaining the lower loss by the UWA method is thought to be the differential urine-soil interactions between methods. Wachendorf *et al.* (2008) reported that 75% of the urine induced $\text{N}_2\text{O}$-N loss in their experiment came from native soil N. It is likely that a significant portion of the urine-induced $\text{N}_2\text{O}$ loss under HL also came from native soil N. A rapid emission peak exceeding 1100 $\mu\text{g} \text{N}_2\text{O}$-N/patch/h was induced from the NEEA simulated patch on the day of application. This peak in emission, occurring at a time when soil TON levels were low (Fig. 2c) and was almost three times larger than the initial peak of 398 $\mu\text{g} \text{N}_2\text{O}$-N/patch/h for the UWA simulated patch (Fig. 3a). In the NEEA method the urine can interact with a greater volume of surface soil as it migrates outwards from the point of application within the collar and tapers off naturally towards the edges. In the case of these experiments the NEEA area was approximately three times larger than the UWA. We suggest that these tapering (Williams & Haynes, 1994) and edge effects could be important because interfaces or edges are often the most active zones of ecosystems. Another factor likely to affect the urine-soil interaction is a degree of transient ponding observed at application in the UWA method. The hydraulic head (Hillel, 2004) created by the artificial urine ponding which occurred in the UWA treatment may have promoted deeper infiltration. Deeper infiltration could reduce $\text{N}_2\text{O}$ production because the nitrification rate in the upper soil layer could be at least an order of magnitude higher than in the lower soil layers (Luo *et al.* 1988). It is also conceivable that ammonia volatilisation loss, an important N loss pathway from urine patches (Fischer *et al*., 2016), could be differentially affected by the patch simulation approach. 

The NEEA method allowed measurement of the naturally occurring patch effective area for the specific soil environmental conditions of this experiment. The 0.462 m$^2$ collar used in the
NEEA method was approximately 3 times larger than the 0.156 m$^2$ collar used in the UWA method. It was larger than the mean wetted area of 0.353 m$^2$ reported for a 2.37 kg urination by Saarijarvi & Virkajarvi (2009) and mean zone of grass response of 0.37 m$^2$ reported by Moir et al. (2010). It was also larger than any of the collars used in previous work listed in Table 1. Anger et al. (2003) accounted for the patch zone of influence to a degree by simulating a 0.2 m$^2$ patch in a 0.24 m$^2$ N$_2$O measurement collar (Table 1) and Rochette et al. (2014) specifically designed their experiment to account for it by simulating 0.1 m$^2$ patches in 0.303 m$^2$ N$_2$O measurement collars.

The method which most closely mimics natural conditions is expected to deliver the most credible quantitative estimate of loss. In the case of these experiments the NEEA mimicked natural conditions much more closely than the UWA method. Although higher loss was recorded for the NEEA method under HL, this may not always be the outcome, for instance no effect was observed under LL. Under different conditions a concentrated zone of N loading as a result of the UWA method could contribute to an elevated NO$_3$-N pool which persists for longer favouring denitrification further from the time of urine application. Some evidence of such an effect is present in the LL data. A significant treatment x day of measurement interaction was detected (P<0.001) with two secondary peaks in emission on days 20 and 30 measured for the UWA method but not for the NEEA method (Fig. 3b). The direction of difference in N$_2$O loss between methods cannot be extrapolated from this study to the diverse soil and environmental conditions in which researchers make N$_2$O loss estimates for urine patches. This manuscript simply highlights a need for greater attention to the method of urine patch simulation. The nature of urine patches raises practical questions of how to best simulate patches for N$_2$O emission measurements. It is suggested that a representative patch can be achieved, at least in part by the following:
a) use of a defined urine volume and N content similar to that of the animal of interest
e.g. close to 2.1 L for dairy cattle (Selbie et al. 2014).

b) allow natural infiltration of the chosen defined volume of urine for the soil of choice
to permit tapering toward the edges as observed in natural patches by Williams &
Haynes (1994).

c) measure from the zone of influence (Saarijarvi & Virkajarvi, 2009) in addition to the
wetted area i.e. the patch effective area (Selbie et al. 2014) or the NEEA.

References

urine patches applied to different N-fertilized swards and estimated annual N₂O
emissions for differently fertilized pastures in an upland location in Germany. Soil
Use and Management, 19(2): 104-111.

Nitrous oxide emissions from cattle excreta applied to a Scottish grassland: Effects of
soil and climatic conditions and a nitrification inhibitor. Science of the Total
Environment, 508: 343-353.

emission of nitrous-oxide from soils. Soil Science Society of America Journal, 46(5):
937-942.

addition on the surface emissions and subsurface concentrations of greenhouse gases
in a UK peat grassland. Agriculture Ecosystems and Environment, 186: 23-32.


**List of Tables**

Table 1. A selection of studies using urine patch simulation for nitrous oxide loss measurement.

Table 2. Effect of the uniform wetted area (UWA) and naturally expanding effective area (NEEA) urine patch simulation methods on N₂O-N loss.
List of Figures

Fig. 1. Precipitation, water filled pore space (WFPS), soil and air temperature during the experiment for a) high loss and b) low loss conditions.

Fig. 2. Soil NH$_4$-N for a) high loss and b) low loss conditions, soil NO$_2$-N and NO$_3$-N [total oxidised N (TON)] for c) high loss and d) low loss conditions (0-10 cm) for naturally expanding effective area (NEEA) and uniform wetted area (UWA) methods.

Fig. 3. Temporal flux of N$_2$O-N emission for a) high loss and b) low loss conditions in response to the uniform wetted area (UWA) and naturally expanding effective area (NEEA) urine patch simulation methods.
Fig. 1. Precipitation, water filled pore space (WFPS), soil and air temperature during the experiment for a) high loss and b) low loss conditions.

Fig. 2. Soil NH$_4$-N for a) high loss and b) low loss conditions, soil NO$_2$-N and NO$_3$-N [total oxidisable N (TON)] for c) high loss and d) low loss conditions (0-10 cm) for naturally expanding effective area (NEEA) and uniform wetted area (UWA) methods. Error bars indicate the pooled standard error of the mean.
Fig. 3. Temporal flux of $N_2O-N$ emission for a) high loss and b) low loss conditions in response to the uniform wetted area (UWA) and naturally expanding effective area (NEEA) urine patch simulation methods.
Pooled standard error of mean = 50.9 μg N/patch/h
Table 1. A selection of studies using urine patch simulation for nitrous oxide loss measurement.

<table>
<thead>
<tr>
<th>Chamber collar size</th>
<th>Patch size</th>
<th>Mean N loading kg N/ha</th>
<th>Mean volume urine in chamber</th>
<th>Urine N content</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>m²</td>
<td>m²</td>
<td>kg N/ha</td>
<td>L per chamber area</td>
<td>L m²</td>
<td>g N/L</td>
</tr>
<tr>
<td>0.1164</td>
<td>0.1164</td>
<td>865, 911</td>
<td>1</td>
<td>8.6</td>
<td>10.07, 10.6</td>
</tr>
<tr>
<td>0.0962</td>
<td>0.0962</td>
<td>1030</td>
<td>1.0</td>
<td>9.9</td>
<td>10.4</td>
</tr>
<tr>
<td>0.1195</td>
<td>0.1195</td>
<td>930</td>
<td>1.1</td>
<td>9.3</td>
<td>10</td>
</tr>
<tr>
<td>0.083</td>
<td>0.083</td>
<td>890 – 3920</td>
<td>1.0, 2.0, 3.0</td>
<td>11.9-35.6</td>
<td>7.5 – 11</td>
</tr>
<tr>
<td>0.24</td>
<td>0.24</td>
<td>425</td>
<td>1.0</td>
<td>4.2</td>
<td>10.2</td>
</tr>
<tr>
<td>0.0875</td>
<td>0.0875</td>
<td>608, 1000</td>
<td>2.5</td>
<td>28.6</td>
<td>14.6, 21.6</td>
</tr>
<tr>
<td>0.2</td>
<td>0.2</td>
<td>300, 500, 700, 1000</td>
<td>2</td>
<td>10</td>
<td>3, 5, 7, 10</td>
</tr>
<tr>
<td>0.0491</td>
<td>0.5</td>
<td>592</td>
<td>0.49</td>
<td>10</td>
<td>5.92</td>
</tr>
<tr>
<td>0.0491</td>
<td>0.5</td>
<td>496-551</td>
<td>0.49</td>
<td>10</td>
<td>4.96-5.51</td>
</tr>
<tr>
<td>0.16</td>
<td>2</td>
<td>498</td>
<td>0.8</td>
<td>5</td>
<td>6.7</td>
</tr>
<tr>
<td>0.0314</td>
<td>0.36</td>
<td>420</td>
<td>1.8</td>
<td>5</td>
<td>8.4</td>
</tr>
<tr>
<td>0.24</td>
<td>0.2</td>
<td>842</td>
<td>2</td>
<td>8.3</td>
<td>10.1</td>
</tr>
<tr>
<td>0.303</td>
<td>0.1</td>
<td>92 - 481</td>
<td>0.9 – 1.4</td>
<td>3.0 – 4.6</td>
<td>3.1 – 10.4</td>
</tr>
<tr>
<td>0.462</td>
<td>0.462</td>
<td>229, 359</td>
<td>2</td>
<td>4.33</td>
<td>5.3, 8.3</td>
</tr>
<tr>
<td>0.156</td>
<td>0.156</td>
<td>679, 1064</td>
<td>2</td>
<td>12.8</td>
<td>5.3, 8.3</td>
</tr>
</tbody>
</table>

Values in italic are calculated from information provided in papers.
Table 2. Effect of the uniform wetted area (UWA) and naturally expanding effective area (NEEA) urine patch simulation methods under high loss (HL) and low loss (LL) conditions on N$_2$O-N loss.

<table>
<thead>
<tr>
<th>Urine patch simulation &amp; measurement method</th>
<th>Patch/collar area (m$^2$)</th>
<th>Urine volume (L/patch)</th>
<th>N load/patch (g N/patch)</th>
<th>Mean N loading (kg N/ha)</th>
<th>N$_2$O-N loss (mg/patch)</th>
<th>Standard deviation (%)</th>
<th>Net emission relative to the NEEA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-NEEA</td>
<td>0.462</td>
<td>2</td>
<td>16.6</td>
<td>359</td>
<td>434.8 a*</td>
<td>156.5</td>
<td>100</td>
</tr>
<tr>
<td>HL-UWA</td>
<td>0.156</td>
<td>2</td>
<td>16.6</td>
<td>1064</td>
<td>280.6 b</td>
<td>65.8</td>
<td>64</td>
</tr>
<tr>
<td>HL-Control</td>
<td>0.156</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>5.5 c</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td>LL-NEEA</td>
<td>0.462</td>
<td>2</td>
<td>10.6</td>
<td>229</td>
<td>35.1 c</td>
<td>10.2</td>
<td>100</td>
</tr>
<tr>
<td>LL-UWA</td>
<td>0.156</td>
<td>2</td>
<td>10.6</td>
<td>679</td>
<td>37.7 c</td>
<td>22.4</td>
<td>108</td>
</tr>
<tr>
<td>LL-Control</td>
<td>0.156</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>3.9 c</td>
<td>3.9</td>
<td>-</td>
</tr>
<tr>
<td>Pooled standard error of the mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>Degrees of freedom</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

* Means with different letters at $P \leq 0.05$.  

...