Universiteit Antwerpen

This item is the archived peer-reviewed author-version of:

Ureolytic activity and its regulation in vibrio campbellii and vibrio harveyi in relation to nitrogen recovery from human urine

Reference:

Defoirdt Tom, Vlaeminck Siegfried, Sun Xiaoyan, Boon Nico, Clauwaert Peter.- Ureolytic activity and its regulation in vibrio campbellii and vibrio harveyi in relation to nitrogen recovery from human urine
Environmental science and technology / American Chemical Society - ISSN 0013-936X - 51:22(2017), p. 13335-13343
Full text (Publisher's DOI): https://doi.org/10.1021/ACS.EST.7B03829
To cite this reference: https://hdl.handle.net/10067/1477030151162165141

uantwerpen.be
Institutional repository IRUA

Environmental Science & Technology

Subscriber access provided by READING UNIV

Article

Ureolytic activity and its regulation in Vibrio campbellii and Vibrio harveyi in relation to nitrogen recovery from human urine

Tom Defoirdt, Siegfried E Vlaeminck, Xiaoyan Sun, Nico Boon, and Peter Clauwaert

Environ. Sci. Technol., **Just Accepted Manuscript** • DOI: 10.1021/acs.est.7b03829 • Publication Date (Web): 30 Oct 2017

Downloaded from http://pubs.acs.org on November 4, 2017

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

ACS Publications

Environmental Science & Technology is published by the American Chemical Society.
1155 Sixteenth Street N.W., Washington, DC 20036
Published by American Chemical Society. Copyright © American Chemical Society.
However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

Ureolytic activity and its regulation in *Vibrio campbellii* and *Vibrio harveyi* in relation to nitrogen recovery from human urine

Tom Defoirdt[1,*], Siegfried E. Vlaeminck[1,2], Xiaoyan Sun[1], Nico Boon[1] and Peter Clauwaert[1]

Author affiliations:

[1] Center for Microbial Ecology and Technology (CMET), Ghent University, Coupure Links 653, 9000 Gent, Belgium

[2] Research Group of Sustainable Energy, Air and Water Technology, Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerpen, Belgium

*Corresponding author: Tom Defoirdt

Address: CMET, Ghent University, Coupure Links 653, 9000 Gent, Belgium

Phone: +32 (0)9 264 59 76

Fax: +32 (0)9 264 62 48

e-mail: Tom.Defoirdt@UGent.be

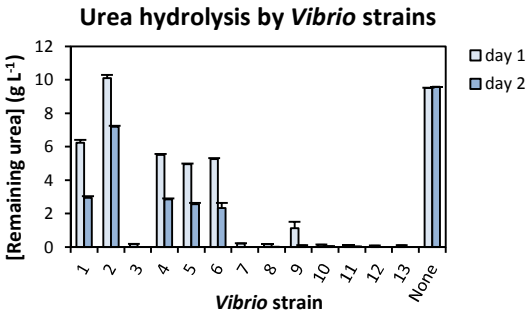
Journal: Environmental Science & Technology

Running title: ureolytic activity by vibrios for nutrient recovery

Key words: urease; marine vibrios; biofilm; motility; indole signaling; blue biotechnology

ABSTRACT

Human urine contains a high concentration of nitrogen and is therefore an interesting source for nutrient recovery. Ureolysis is a key requirement in many processes aiming at nitrogen recovery from urine. Although ureolytic activity is widespread in terrestrial and aquatic environments, very little is known about the urease activity and regulation in specific bacteria other than human pathogens. Given the relatively high salt concentration of urine, marine bacteria would be particularly well suited for biotechnological applications involving nitrogen recovery from urine, and therefore, in this study, we investigated ureolytic activity and its regulation in marine vibrios. Thirteen out of 14 strains showed ureolytic activity. The urease activity was induced by urea, since complete and very rapid hydrolysis – up to 4 grams per liter per hour (g L^{-1} h^{-1}) of urea – was observed in synthetic human urine when the bacteria were pretreated with 10 g L^{-1} urea, whereas slow hydrolysis occurred when they were pretreated with 1 g L^{-1} urea (14-35% hydrolysis after 2 days). There was no correlation between biofilm formation and motility on one hand, and ureolysis on the other hand, and biofilm and motility inhibitors did not affect ureolysis. Together, our data demonstrate for the first time the potential of marine vibrios as fast urea hydrolysers for biotechnological applications aiming at nutrient recovery from human urine.



INTRODUCTION

Human urine contains relatively high concentrations of urea, sodium, chloride, phosphate and potassium, and trace levels of calcium, sulfate and magnesium[1]. Therefore, urine is an interesting source of nutrients for recovery, both in conventional applications such as the treatment of source-separated urine[2] and more futuristic applications such as the Micro-Ecological Life Support System Alternative (MELiSSA) of the European Space Agency (ESA)[3]. As most nitrogen recovery pipelines act upon ammonical nitrogen, urease (urea amidohydrolase) is a key enzyme for nitrogen recovery from urine as it catalyzes the hydrolysis of urea into ammonia and carbamic acid, the latter of which spontaneously decomposes into carbonic acid and a second ammonia molecule[4]. It can take more than a month to achieve complete urea hydrolysis when one relies only on indigenous ureolytic bacteria present in source-separated urine, and this is too slow for practical purposes[5]. In collecting pipes and collection tanks of urine-collecting toilets, complete hydrolysis has been obtained within a few days because of the relatively high bacterial densities (e.g. in biofilms on the pipes and tanks)[6]. Hence, the use of urease-producing bacteria can significantly improve the efficiency of the urea hydrolysis process. Further optimization of this process will thus lead to shorter incubation times needed to achieve complete hydrolysis, thereby enabling the use of smaller urine collection tanks.

Urease activity and its regulation have been extensively studied in human (urinary tract) pathogens such as *Proteus mirabilis, Helicobacter pylori* and *Ureaplasma urealyticum* in relation to the pathogenicity process[7]. The urease gene cluster usually consists of structural genes (encoding the urease subunits) as well as accessory genes. The accessory genes encode enzymes that are required for the generation of catalytically active urease, mainly by facilitating the assembly of the nickel metallocenter within the active site of the enzyme[8]. Three regulatory patterns have been described for urease activity: constitutive, induced by urea and repressed by nitrogen-rich compounds (most notably ammoniacal

nitrogen)[7]. Although urease activity is known to be widespread in terrestrial and aquatic environments[9], very little is known on the urease activity and regulation in bacteria other than human pathogens. This knowledge is particularly important with respect to highly controlled life support systems such as MELiSSA that require to work with a well-defined microbial community. Given the fact that urine contains a relatively high salt concentration (approximately 14 g L^{-1})[2], marine bacteria, which are naturally tolerant to relatively high pH (>8 in the ocean) and high salt concentrations (on average 35 g L^{-1} in the marine environment[10]), would be particularly well suited for biotechnological applications involving nitrogen recovery from urine. However, a detailed analysis of ureolytic activity and its regulation in specific marine bacteria is currently lacking.

Urea in aquatic ecosystems comes from protein catabolism, excretion by plankton and macrofauna (e.g. the excretion of urine by marine mammals). Although urea is generally present at low concentrations in the bulk marine environment, on a local (micro-)scale, marine microbes can encounter significantly higher concentrations[11]. Vibrios (including species belonging to the *Harveyi* clade or core group, such as *Vibrio campbellii* and *Vibrio harveyi*) are amongst the dominant heterotrophic bacteria in marine environments, showing a remarkable metabolic versatility, and the capability to move to and exploit nutrient hot spots[12]. *Vibrio campbellii* and *Vibrio harveyi* can be found both free-living in the water column and in association with other marine organisms, including algae, mollusks, crustaceans and finfish[13]. Urease activity has been reported in vibrios, most notably in the human pathogen *Vibrio parahaemolyticus*, for which the urease gene cluster has been identified[14]. Other, non-human pathogenic species belonging to the *Harveyi* clade are also known to contain ureolytic strains, and presence of ureolytic activity is one of the phenotypic characteristics used in taxonomy of these bacteria[15-16]. Depending on the study, roughly 50-70% of the *Vibrio campbellii* and *Vibrio harveyi* strains have been reported as urease positive. Further, the genome sequenced *Vibrio harveyi* strain ATCC 35084 contains homologs to all genes from the urease cluster of *Vibrio parahaemolytics* (**SI Figure 1**). Hence, in

order to optimise nitrogen recovery from urine, in this study, we aimed at determining the ureolytic capability and its regulation in marine bacteria, using *Vibrio campbellii* and *Vibrio harveyi* as model organisms.

MATERIALS AND METHODS

Strains and growth conditions. Strains used in this study are listed in **Table 1**. The strains were routineously grown in Luria-Bertani (LB) broth (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}) or marine LB broth (m-LB; tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, Instant Ocean synthetic sea salt 35 g L^{-1}). Cell densities were determined spectrophotometrically at 600 nm.

Synthetic urine. Synthetic urine was prepared as described previously[20-21] , with some modifications (**SI Table 1**). Specifically, acetate was used as carbon source in order to reduce complexity of the medium, and phosphate levels were increased to levels that are more similar to those found in urine[6].

Determination of urea and ammonium. Urea and ammonium were determined colorimetrically based on reaction with Ehrlich's reagent[22] and the Berthelot reaction[23], respectively. Urea levels were proportional to OD430 in the range 0-1500 mg L^{-1} (OD430 = 0.0017 x [urea]; R^2 = 0.9997) and ammonium levels were proportional to OD695 in the range 0-5 mg L^{-1} (OD695 = 0.5291 x [NH_4^+]; R^2 = 0.9741).

Screening of urea hydrolysis in Christensen's urea broth. The capability of the strains to hydrolyse urea was first screened using Christensen's urea broth. The strains were grown in m-LB to an OD600 of 1

(corresponding to approximately 10^9 cells mL^{-1}), re-inoculated (1% v/v) into test tubes containing 3 mL Christensen's urea broth (**SI Table 2**) and incubated at 28°C for 2 days. Christensen's broth contains the pH indicator phenol red, which gives the medium a pink color at elevated pH levels (pink at pH > 8.2; yellow at pH < 6.8). Ureolysis will increase the pH of the broth, which will thus be manifested by a pink color. For each strain, a control test was performed in the same medium without urea, in order to confirm that the pH increase was indeed due to ureolysis.

Screening of urea hydrolysis in a nutrient-rich background. In order to confirm the results obtained in Christensen's broth, a second screening was performed. Synthetic urine was mixed with m-LB; in a 1:1 ratio. The strains were inoculated into this medium (1% v/v), and after 3 days of incubation at 28°C, cell densities and remaining urea levels were determined.

Urea hydrolysis in synthetic urine. In a subsequent experiment, the urea hydrolysis capabilities of the strains were quantified in synthetic urine. The strains were grown for 24h in LB broth to OD600 of 1, after which they were inoculated (1% v/v) in triplicate into fresh synthetic urine medium (**SI Table 1**). After 1 and 2 days of incubation at 28°C with shaking (150 rpm; tubes incubated horizontally), cell densities and remaining urea levels were determined.

Screening of urea hydrolysis in diluted synthetic urine media. In order to investigate the impact of the salt concentration and urea level on urea hydrolysis, the urea hydrolysis capabilities of the strains were screened in diluted synthetic urine. Four diluted synthetic urine media were prepared: threefold diluted in distilled water, threefold diluted in a salts solution containing all salts present in the synthetic urine (**SI Table 3**), and tenfold diluted in the salts solution. The isolates were grown for 24h in LB broth,

after which they were inoculated (1% v/v) into diluted synthetic urine media. The suspensions were incubated for 2 days at 28°C without shaking. After incubation, cell densities, pH, remaining urea and ammonia levels (threefold diluted media only) were determined. Conductivity of the supernatants was measured with an electrochemical analyser (Consort C6010).

Urea hydrolysis in buffered threefold diluted synthetic urine. *Vibrio harveyi* LMG 22893, *Vibrio campbellii* LMG 22895 and *Vibrio harveyi* E022 were selected for a more in depth examination of the degradation of urea in diluted urine. The selected strains were grown overnight in LB broth at 28°C to an OD600 of 1, after which the grown cultures were re-inoculated (1% v/v) in triplicate into synthetic urine that was diluted threefold with 75 mM phosphate buffer (pH 7) (thus giving a final concentration of 50 mM). After 1, 2 and 7 days of incubation at 28°C, cell density, remaining urea levels, ammonium levels, pH and conductivity were measured.

Regulation of urea hydrolysis by urea and ammonium. In order to investigate the impact of urea and ammonium levels on urea hydrolysis in the selected strains, a pretreatement experiment was performed, in which the strains were pretreated with different concentrations of urea and/or ammonium, after which urea hydrolysis was quantified in synthetic urine. *Vibrio harveyi* LMG 22893, *Vibrio campbellii* LMG 22895 and *Vibrio harveyi* E022 were grown for 24h in LB broth, after which they were inoculated (1% v/v) in triplicate into 2 ml Eppendorf tubes containing 1 mL urine salts solution (**SI Table 3**) in 100 mM phosphate buffer (pH 7) and supplemented with different concentrations of urea (0, 1 and 10 g L^{-1}, respectively) and/or ammonium (0, 1 and 10 g L^{-1}, respectively), which was added as NH_4Cl in order to avoid pH changes. After overnight incubation, the suspensions were centrifuged (5 min 5000 x g), and cells were resuspended in 1 mL volumes of synthetic urine. Erythromycin (50 mg L^{-1}) was added in order to prevent *de novo* synthesis of urease in the synthetic urine. The cultures were

incubated statically at 28°C, and every 2h, the tubes were vortexed and samples were taken to determine the remaining urea levels.

Determination of biofilm levels. Biofilm formation by the strains was investigated in order to determine whether there is a relation between biofilm formation and urea hydrolysis. The strains were grown overnight in LB or m-LB broth at 28°C, after which the grown cultures were re-inoculated (1% v/v) into fresh medium (LB, m-LB, or buffered threefold diluted synthetic urine). Two hundred microliter aliquots of the suspensions were transferred to the wells of a transparent 96-well plate (6 replicates per strain). The plate was incubated at 28°C with shaking (100 rpm), after which planktonic cell density was measured (OD600). Biofilm levels were determined by crystal violet staining, as described previously[24]. Briefly, the wells were rinsed twice with tap water to remove non-adherent cells, after which biofilms were stained for 20 min with a 1 g L^{-1} crystal violet solution. The wells were subsequently rinsed three times with tap water, plates were air dried, and stained biofilms were redissolved in ethanol, after which absorbance of crystal violet (OD540) was measured.

Determination of motility. Motility of the strains was investigated in order to determine whether there is a relation between this phenotype and urea hydrolysis Motility was determined as described previously[25]. Briefly, the strains were grown overnight in LB broth at 28°C, after which 5 µL aliquots were applied to the centre of soft LB agar plates (LB broth with 0.2% agar). The plates were incubated upright at 28°C for 24h, after which the motility zone was measured.

Regulation of ureolysis by indole signaling and motility. In order to further investigate whether there is a relation between urea hydrolysis on one hand, and biofilm formation or motility on the other

hand, activity experiments were performed in which the impact of inhibitors of these phenotypes on urea hydrolysis was determined. The strains were incubated overnight in a salts solution containing 10 g L^{-1} urea, 100 mM phosphate buffer, with or without indole or the motility inhibitor phenamil at 200 µM and 50 µM, respectively[25-26]. All compounds were dissolved in DMSO, and equal volumes of DMSO were added to all treatments. Ureolytic activity was further determined in synthetic urine supplemented with erythromycin as described higher.

Statistics. All statistical analyses were performed with the SPSS software, version 23.

RESULTS AND DISCUSSION

Screening of ureolytic activity of *Vibrio campbellii* and *Vibrio harveyi* strains. Vibrios are known to have a large metabolic versatility, and are able to quickly exploit nutrient hot spots in the marine environment[12-13]. Moreover, as marine organisms, they are able to cope with high salt concentrations (which are approximately threefold higher in the marine environment than in urine). Therefore, in this study, we aimed at determining the abilities of *Vibrio campbellii* and *Vibrio harveyi* strains to quickly hydrolyse urea. As a first screening test, the vibrios were grown in Christensen's broth, which contains the pH indicator phenol red, giving the medium a pink color at pH values above 8.2 (which is the case if urea is hydrolised into ammonium). All strains, except for strain BB120, induced a color change in Christensen's broth containing urea, whereas none of them caused a color change in the absence of urea. Hence, all strains (except for BB120) are capable of hydrolysing urea. The fact that strain BB120 did not show ureolytic activity is consistent with the absence of homologs of the urease gene cluster in its genome sequence (GenBank accession n° CP006605 and CP006606).

In order to confirm these screening results, the strains were inoculated into a 1:1 mixture of synthetic urine and marine LB (m-LB) broth, and cell densities and remaining urea levels were determined after 3 days of incubation. Consistent with the results obtained in Christensen's urea broth, all strains, except for BB120, were able to hydrolyse urea, as manifested by a decrease in the urea concentration in the medium (**SI Figure 2**). Based on its incapability to hydrolyse urea, strain BB120 was excluded from further experiments.

Urea hydrolysis in synthetic urine. Our previous experiment indicated that most of our *Vibrio campbellii* and *Vibrio harveyi* strains are able to hydrolyse urea. In a further experiment aiming at a more quantitative evaluation of the ureolytic activity, the strains were inoculated into synthetic urine (containing 10 g L^{-1} of urea, which is within the range of what is found in fresh urine[2,6]), and cell densities and urea levels were determined after 1 and 2 days of incubation at 28°C. All strains showed ureolytic activity, although there was considerable variability between strains (**Figure 1A**). Several of the strains completely hydrolysed the urea in one or two days. Others showed partial hydrolysis from 10 to approximately 3 g L^{-1} after 2 days, whereas strain LMG 21362 showed relatively poor hydrolysis (more than 7 g L^{-1} left at day 2). We think that this reflects differences between the strains with respect to urea hydrolysis rates, since there were no clear-cut differences in cell densities between strains that showed complete hydrolysis and those that showed partial hydrolysis. Although we stopped the experiment after 2 days, we assume that the latter would also have completely hydrolysed the urea after a prolonged incubation. At day 2, the pH of the medium was in the range 9.2-9.3 for all strains (which is consistent with the formation of an ammonia buffer by urea hydrolysis), whereas it had remained at 7.7 in the sterile control. Most of the strains (except for LMG 21362) were growing relatively poorly in the urine medium (maximum OD_{600} of 0.01-0.2 – compared to 0.8-1.5 in m-LB), with densities at day 1 being slightly higher than at day 2 (**Figure 1B**). The relatively poor growth might relate to the release of

ammonia, thereby increasing the pH of the medium. Indeed, although these bacteria can tolerate high pH[27], the optimal pH for growth is around 7[28]. A reduced growth might be advantageous for biotechnological applications as in this way, excessive sludge production would be avoided. On the other hand, growth should be strong enough in order to maintain sufficient biomass in long-running urea hydrolysis or urine nitrification reactors in order to maintain urea hydrolysis at the desired level.

Screening of urea hydrolysis capability in diluted synthetic urine media. In order to further characterise the ureolytic activity of the strains, a further screening was performed in diluted synthetic urine. We especially paid attention to the urea level (which has previously been reported to affect ureolysis rates in some bacteria[7]) and the salt concentration. We specifically aimed at testing urea hydrolysis in diluted urine as this is relevant for urine collection systems in which urine is diluted with flush water. Although all strains are capable to degrade urea (as manifested in undiluted synthetic urine), the hydrolysis rates were lower in the diluted media (**SI Table 4**). In general, the lower the urea levels at the start of the incubation, the lower the hydrolysis rates: 1.4-9.9 g urea L^{-1} day^{-1} at 10 g L^{-1} urea, 0.1-1.7 g urea L^{-1} day^{-1} at 3.3 g L^{-1} and 0.0-0.1 g L^{-1} day^{-1} at 1 g L^{-1} urea (all at the same salt concentration). Differences were significant when comparing the rates at 1 g L^{-1} urea to those at 10 g L^{-1} (independent samples t-test; $P < 0.001$) and 3.3 g L^{-1} ($P < 0.001$). This suggested that the urease of *Vibrio campbellii* and *Vibrio harveyi* is induced by urea, and this is one of the regulatory mechanisms that have been described in other ureolytic bacteria[7]. When comparing the two threefold dilutions, the hydrolysis was faster in the dilution in the salts solution than in the dilution in distilled water (independent samples t-test; $P = 0.012$). This might reflect the marine origin of the strains, implying that they have evolved to have maximal activity at higher salt concentrations. Finally, in the tenfold dilution, no or poor hydrolysis was observed (**SI Table 4**).

There was considerable variation amongst the strains with respect to the final cell density after 2 days (**SI Table 4**). There was a strong, positive correlation between ureolysis and pH, and a strong negative correlation between pH and cell density, except for the tenfold dilution (**Table 2**). This suggests that the pH increase following from ureolysis decreased the growth of the vibrios. Various levels of a 500 mM phosphate buffer solution (pH 7.0) were added to the media in which ureolysis had occurred in order to determine the buffer concentrations needed to neutralize the pH increase in further experiments (**SI Table 5**).

Urea hydrolysis in buffered threefold diluted synthetic urine. The results of the screening in diluted synthetic urine media suggested that ureolysis is induced by urea (> 1 g L^{-1}) in *Vibrio campbellii* and *Vibrio harveyi*. Strains LMG 22893, LMG 22895 and E022 (showing different behaviours in diluted urine media) were selected for a more in-depth examination of the urease activity and regulation, using threefold diluted urine. The diluted urine was buffered with phosphate buffer (final concentration 50 mM, pH 7.0) in order to exclude the possibility that differences in urea hydrolysis could be caused by differences in pH. Strains LMG 22893 and LMG 22895 showed complete hydrolysis of urea within 2 days, whereas strain E022 did not show ureolysis, even after 7 days (**Figure 2A**). In addition to direct determination of urea, ammonium levels were also measured, and based on both measurements, the urea levels that were hydrolysed and the ammonium levels that were formed were calculated, respectively. In general, there was a good match between both measurements (**SI Figure 3**). Even in the presence of a 50 mM phosphate buffer, the ureolysis resulted in a slightly increased pH (from 7 to 8) (**Figure 2B**), and an increased conductivity was observed in the cultures of strains LMG 22893 and LMG 22895 (from roughly 20 to 30 mS cm^{-1}; **Figure 2C**). All three strains showed clear growth in this medium (**Figure 2D**), which is in contrast to unbuffered urine, where in general poor growth was observed (**SI Table 4**).

Further, a short-term experiment in undiluted and threefold diluted synthetic urine (either in distilled water or phosphate buffer, pH 7, final concentration 50 mM) was performed. No ureolysis was observed until 4h, at which time point ureolysis had started in strain E022 incubated in undiluted urine (**Figure 3**). After 6h, all three strains showed ureolysis in undiluted urine. However, no ureolysis was observed yet for any of the strains in the threefold diluted synthetic urine media. These results indicated that induction of ureolysis by urea is not a peculiarity of strain E022 as it was observed for all three strains. In contrast to this, induction by urea has not been observed by Tang *et al.*, who found that ureolysis rates of an undefined ureolytic enrichment culture from seawater were not affected by dilution of urine in seawater (up to tenfold dilution)[5]. Hence, in contrast to *Vibrio campbellii* and *Vibrio harveyi*, ureolysis in other marine bacteria apparently is not induced by urea.

Impact of urea and ammonium concentrations on ureolytic activity. In order to further substantiate the induction of urease by urea, and to investigate the role of ammonium, further activity experiments were performed. In these experiments, the bacteria were incubated overnight in urine salts solution (in 100 mM phosphate buffer, pH 7) and supplemented with different levels of urea and/or ammonium. After incubation, the suspensions were centrifuged, the salts solutions discarded and undiluted synthetic urine was added. In order to avoid confusion due to the activation of urease by the urea present in the synthetic urine during assessment of the ureolytic activity, erythromycin was added to block protein synthesis (and in this way prevent *de novo* synthesis of urease in the synthetic urine medium). Cultures pretreated with 10 g L^{-1} urea showed rapid ureolysis, with more than 60% hydrolysis after 2h, and (almost) complete hydrolysis after 4h (**Figure 4**). Consistent with urease induction by urea, no urease activity was detected in cultures that were pretreated with a salts solution without urea or with 1 g L^{-1} urea. This also confirmed the inhibition of *de novo* urease synthesis in the synthetic urine by the addition of erythromycin – compare the 6h data points in **Figure 4** (urea 0 + NH_4 0) to those of

Figure 3 (undiluted urine). In order to determine whether urease activity was indeed absent or rather very low in cultures of LMG 22893 and LMG 22895, remaining urea levels were again measured after 2 days of incubation. The results suggest that there was low urease activity in cultures pretreated with a salts solution containing 1 g L^{-1} urea (14-35% removal), whereas ureolysis was only observed for strain LMG 22895 in cultures that were pretreated with a salts solution without urea (5% removal – **SI Figure 4**). Ureolysis rates were calculated and were found to be more than 50-fold higher if cells were pretreated with 10 g L^{-1} urea when compared to cells that were pretreated with 1 g L^{-1} urea (**Table 3**). The ureolysis rates of cells exposed to 10 g L^{-1} urea are 5- to 25-fold higher than those reported by Udert *et al.*, where urea was hydrolysed in source-separated urine by indigenous micro-organisms in collection pipes and tanks of urine-collecting toilets (with hydrolysis rates of 162-880 mg urea L^{-1} h^{-1})[6]. The time to achieve complete ureolysis was 10 times shorter than what Liu *et al.* reported for fresh urine that was mixed with completely hydrolysed urine (as a source of urease)[29], and comparable to what Tang *et al.* reported for an enrichment culture obtained from seawater after 4 cycles of cultivation in a 1:1 mixture of seawater and urine (although no complete hydrolysis was obtained in the latter study)[5]. These data indicate that inoculation of urine with ureolytic marine bacteria (such as *V. campbellii* and *V. harveyi*) is a promising strategy to increase the ureolysis efficiency and in this way decrease the incubation time needed to achieve complete hydrolysis, and the volumes of urine storage tanks.

Another regulatory mechanism that has been described in some ureolytic bacteria is repression by other nitrogen sources (most notably ammoniacal nitrogen)[7]. However, we found that the pretreatment of *V. campbellii* or *V. harveyi* with ammonium did not affect ureolysis, whereas ammonium even increased the ureolytic activity in the presence of 10 g L^{-1} urea (**Figure 4** – only results for pretreatment with 10 g L^{-1} ammonium are shown; pretreatment with 1 g L^{-1} ammonium resulted in a similar, but less pronounced response).

The fact that *V. campbellii* and *V. harveyi* are capable of quickly converting urea to ammonium, and that this activity is only induced at relatively high urea concentrations (g L^{-1} range), is consistent with the reputation of these bacteria for being copiotrophs[30]. Despite the superficially homogeneous appearance of the marine water column, nutrients are not homogeneously distributed at scales relevant to micro-organisms but rather occur as hot spots since they are often associated with or released from particles such as micro-algae, fecal pellets and marine snow[12], and these are also the sites where relatively high urea levels can be expected[11]. In order to cope with the conditions that prevail in the marine environment (i.e. low nutrient levels in the bulk and high levels in hot spots), marine bacteria have evolved two divergent strategies: they are either (1) minute, nonmotile, with a streamlined genome, or (2) relatively large, motile, with a high metabolic flexibility and a large genome[12]. The second group is adapted to exploit the relatively rare resource-rich conditions, and chemotactic motility enables them to access novel, nutrient-rich hot spots[12]. Bacteria belonging to the *Vibrionaceae* family belong to this group: they are often (highly) motile, have large genomes (2 chromosomes), show a high metabolic flexibility and are capable of quickly increasing their population size if conditions are favorable[30].

Relation between ureolysis and biofilm formation. Biofilms have previously been implicated in urease activity in bacteria[5,31]. Therefore, the biofilm formation capability of the *Vibrio campbellii* and *Vibrio harveyi* isolates was determined in m-LB broth, and all strains showed clear biofilm formation (**SI Figure 5**). We calculated correlations (Spearman's rho) between the biofilm formation capability of the strains (data from **SI Figure 5**) and the urea removal in different media (data from **Figure 1** and **SI Table 4**) in order to determine whether there is a link between both phenotypes. However, no significant correlations were observed.

In a further experiment, biofilm formation of the three selected strains (LMG 22893, LMG 22895 and E022) was determined in buffered threefold diluted synthetic urine in 96-well plates. LB medium

(containing approximately the same salt concentration) was used as a reference. The vibrios showed growth in both media, which was higher in LB than in the diluted synthetic urine (**Figure 5A and B**). The growth was much lower than in m-LB under the same conditions (compare to **SI Figure 5A**) and than growth in buffered threefold diluted urine in test tubes incubated horizontally on a shaker (compare to **Figure 2A**).

No biofilm could be observed for cultures incubated in diluted synthetic urine (**Figure 5C**), whereas slight biofilm formation was observed for cultures incubated in LB medium, especially after 7 days (**Figure 5D**). However, the biofilm levels were considerably lower than in m-LB medium (compare with **SI Figure 5B**). Given the fact that complete ureolysis was already observed after 2 days in buffered threefold diluted urine for strains LMG 22893 and LMG 22895, these observations suggest that biofilm formation is not required for ureolytic activity.

In order to further investigate the impact of the biofilm phenotype on ureolytic activity, we determined the impact of pretreatment with indole on ureolytic activity of *Vibrio harveyi* LMG 22893 and *Vibrio campbellii* LMG 22895. The addition of indole has recently been shown to block biofilm formation in these bacteria, with a 5-fold decrease in biofilm levels in the presence of 200 µM indole[26]. The bacteria were pretreated with a buffered salts solution containing 10 g L^{-1} urea, with or without indole (200 µM), and the urease activity was monitored in synthetic urine supplemented with erythromycin. Similar to the previous experiment (**Figure 4**), rapid ureolysis was observed in cultures that were pretreated with urea, whereas no ureolysis occurred in cultures that were not pretreated with urea (**Figure 6**). Further, indole had no impact on the activity, again indicating that the biofilm phenotype (and other phenotypes controlled by indole) does not affect ureolysis.

Relation between ureolysis and motility. Motility is another phenotype that has been correlated with urease activity[32]. Since vibrios are known to be highly motile bacteria, we aimed at determining the relation between motility and ureolysis. First, we determined the motility capability of all strains on soft LB agar. Although there was considerable variation between strains (Coefficient of variation = 0.44), all of them showed to be motile, with motility zones roughly ranging between 10 and 60 mm (**SI Figure 6**). We calculated Spearman's correlations between the motility of the strains (data from **SI Figure 6**) and the urea removal in different media (data from **Figure 1** and **SI Table 4**) in order to obtain a first indication on whether both phenotypes are linked. However, no significant correlations were observed. Unfortunately, due to the poor growth in synthetic urine media, it is not possible to determine motility of the strains in this matrix.

In order to further investigate the link between motility and ureolysis, a chemical biological approach was used, involving pretreatment of the bacteria with urea and the specific motility inhibitor phenamil[25]. Rapid ureolysis was observed for bacteria pretreated with urea and phenamil (although slightly slower than for bacteria pretreated with urea alone) (**Figure 6**). These data indicate that motility does not have a major impact on ureolysis and this is also consistent with indole having no impact on ureolytic activity, since indole has also been shown to block motility in these bacteria (3-fold decrease in the presence of 200 µM indole[26]).

Further perspectives. In this study, we investigated the potential of marine vibrios for faster hydrolysis of source-separated urine, and found that several strains completely hydrolysed the urea present in synthetic urine within one day. The ureolysis was 10-fold faster than what has previously been reported for indigenous micro-organisms in source-separated urine, indicating that the use of ureolytic marine vibrios indeed holds promise as a strategy to increase the rate of urea hydrolysis. Hence, inoculation of urine storage tanks with vibrios could be a valid strategy to decrease the incubation time needed to

achieve complete hydrolysis, thereby enabling the use of smaller urine storage tanks. Further research is needed in order to investigate the impact of inoculating urine with vibrios on downstream processes (e.g. coupling with nitrification, which would be needed, for instance, in bioregenerative life support systems) and to investigate whether vibrios can maintain themselves in urine-fed reactors (either or not diluted and either or not pH buffered) in case these would need to be operational over a longer period. Although biofilm formation did not affect ureolysis in our experiments, it might contribute to the survival of vibrios in urine reactors, and thereby contribute to long-term activity. Our further research will aim at investigating whether complete nitrification of urine can be obtained by vibrios and nitrifiers, either in a 1-step process (all bacteria in one reactor) or a 2-step process (composed of a hydrolysis reactor, the effluent of which is fed to a nitrifying reactor). Our observation that ureolysis in *V. campbellii* and *V. harveyi* is induced by urea, whereas it is not inhibited by ammoniacal nitrogen, indicates that the urea level is the main parameter that can be used to steer the ureolytic activity of vibrios, thus suggesting that the most effective ureolysis will occur in (sequencing) batch (high initial urea levels) rather than continuous (lower urea levels) set-ups.

ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

SI Table 1 shows the composition of the synthetic urine medium. SI Table 2 shows the composition of Christensen's urea broth. SI Table 3 shows the composition of the urine salts solution. SI Table 4 contains the screening data of urea degradation by the different strains in diluted synthetic urine media. SI Table 5 shows the pH of hydrolysed urine media after addition of various levels of phosphate buffer.

SI Figure 1 shows a schematic representation of the urease gene clusters of *Vibrio harveyi* and *Vibrio parahaemolyticus*. SI Figure 2 shows cell densities and urea levels in 50% m-LB – 50% synthetic urine after 3 days of incubation with different *V. campbellii* and *V. harveyi* strains. SI Figure 3 shows the degraded urea and formed ammonium levels in the cultures of *Vibrio harveyi* LMG 22893, *Vibrio campbellii* LMG 22895 and *Vibrio harveyi* E022 in buffered threefold diluted synthetic urine. SI Figure 4 shows the remaining urea levels in synthetic urine containing pretreated *Vibrio harveyi* LMG 22893 and *Vibrio campbellii* LMG 22895, after 2 days incubation. SI Figure 5 shows planktonic growth and biofilm levels of the *V. campbellii* and *V. harveyi* strains in marine LB broth. SI Figure 6 shows the motility of the *V. campbellii* and *V. harveyi* strains on soft LB agar.

AUTHOR INFORMATION

Corresponding author

*(T.D.) Phone: +32 (0)9 264 59 76; fax: +32 (0)9 264 62 48; e-mail: Tom.Defoirdt@UGent.be

Notes

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

This article has been made possible through the authors' involvement in the URINIS project (RFQ/3-14651/16/NL/JK), as part of MELiSSA, ESA's life support system research program

(http://www.esa.int/Our_Activities/Space_Engineering_Technology/Melissa). We kindly acknowledge the financial support of the Belgian Science Policy (BELSPO).

REFERENCES

(1) Larsen, T.A.; Gujer, W. Separate management of anthropogenic nutrient solutions (human urine). *Water Sci. Technol*. **1996,** *34*, 87-94.
(2) Maurer, M.; Pronk, W.; Larsen, T.A. Treatment processes for source-separated urine. *Water Res*. **2006,** *40*, 3151-3166.
(3) Godia, F.; Albiol, J.; Montesinos, J.L.; Perez, J.; Creus, N.; Cabello, F.; Mengual, X.; Montras, A.; Lasseur, C. MELISSA: a loop of interconnected bioreactors to develop life support in space. *J. Biotechnol*. **2002,** *99*, 319-330.
(4) Mobley, H.L.T.; Hausinger, R.P. Microbial ureases: significance, regulation, and molecular characterization. *Microbiol. Rev.* **1989,** *53*, 85-108.
(5) Tang, W.T.; Dai, J.; Liu, R.; Chen, G.H. Microbial ureolysis in the seawater-catalysed urine phosphorus recovery system: kinetic study and reactor verification. *Water Res.* **2015,** *87*, 10-19.
(6) Udert, K.M.; Larsen, T.A.; Biebow, M.; Gujer, W. Urea hydrolysis and precipitation dynamics in a urine-collecting system. *Water Res*. **2003,** *37*, 2571-2582.
(7) Mobley, H.L.T.; Island, M.D.; Hausinger, R.P. Molecular biology of microbial ureases. *Microbiol. Rev*. **1995,** *59*, 451-480.
(8) Carter, E.L.; Flugga, N.; Boer, J.L.; Mulrooney, S.B.; Hausinger, R.P. Interplay of metal ions and ureases. *Metallomics* **2009,** *1*, 207-221.
(9) Krajewska, B. Ureases I. Functional, catalytic and kinetic properties: a review. *J. Mol. Cat. B: Enzymatic* **2009,** *59*, 9-21.
(10) Sverdrup, H.U.; Johnson, M.W.; Fleming, R.H. (eds.) The Oceans, their physics, chemistry, and general biology. **1942**, Prentice Hall, New York.
(11) Solomon, C.M.; Collier, J.L.; Berg, G.M.; Glibert, P.M. Role of urea in microbial metabolism in aquatic systems: a biochemical and molecular review. *Aquat. Microb. Ecol*. **2010,** *59*, 67-88.
(12) Stocker, R. Marine microbes see a sea of gradients. *Science* **2012,** *338*, 628-633.
(13) Thompson, F.L.; Iida, T.; Swings, J. Biodiversity of vibrios. *Microbiol. Mol. Biol. Rev*. **2004,** *68*, 403-431.
(14) Park, K.S.; Iida, T.; Yamaichi, Y.; Oyagi, T.; Yamamoto, K.; Honda, T. Genetic characterization of DNA region containing the *trh* and *ure* genes of *Vibrio parahaemolyticus. Infect. Immun*. **2000,** *68*, 5742-5748.
(15) Gauger, E.J.; Gomez-Chiarri, M. 16S ribosomal DNA sequencing confirms the synonymy of *Vibrio harveyi* and *V. carchariae. Dis. Aquat. Org*. **2002,** *52*, 39-46.
(16) Lal, M.T.B.M.; Ransangan, J. Taxonomic classification of *Vibrio harveyi* using 16S rDNA and atpA gene sequencing method. *Int. J. Res. Pure Appl. Microbiol*. **2013,** *3*, 17-24.
(17) Defoirdt, T.; Crab, R.; Wood, T.K.; Sorgeloos, P.; Verstraete, W.; Bossier, P. Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi, Vibrio campbellii* and *Vibrio parahaemolyticus. Appl. Environ. Microbiol.* **2006,** *72*, 6419-6423.
(18) Robertson, P.A.W.; Calderon, J.; Carrera, L.; Stark, J.R.; Zherdmant, M.; Austin, B. Experimental *Vibrio harveyi* infections in *Penaeus vannamei* larvae. *Dis. Aquat. Org*. **1998,** *32*, 151-155.
(19) Zhang, X.H.; Meaden, P.G.; Austin, B. Duplication of hemolysin genes in a virulent isolate of *Vibrio harveyi. Appl. Environ. Microbiol*. **2001,** *67*, 3161-3167.
(20) Brooks, T.; Keevil, C.W. A simple artificial urine for the growth of urinary pathogens. *Lett. Appl. Microbiol*. **1997,** *24*, 203-206.

(21) Kim, W.; Tengra, F.K.; Young, Z.; Shong, J.; Marchand, N.; Chan, H.K.; Pangule, R.C.; Parra, M.; Dordick, J.S.; Plawsky, J.L.; Collins, C.H. 2013. Spaceflight promotes biofilm formation by *Pseudomonas aeruginosa. PONE* **2013,** *8*, e62437.
(22) Yatzidis, H.; Garidi, M.; Vassilikos, C.; Mayopoulou, D.; Akilas, A. An improved method for the simple and accurate colorimetric determination of urea with Ehrlich's reagent. *J. Clin. Path*. **1964,** *17*, 163-164.
(23) Bucur, B.; Catala Icardo, M.; Martinez Calatayud, J. Spectrophotometric determination of ammonium by an rFIA assembly. *Rev. Roum. Chim*. **2006,** *51*, 101-108.
(24) Yang, Q.; Anh, N.D.Q.; Bossier, P.; Defoirdt, T. Norepinephrine and dopamine increase motility, biofilm formation, and virulence of *Vibrio harveyi. Front. Microbiol*. **2014,** *5*, 584.
(25) Yang, Q.; Defoirdt, T. Quorum sensing positively regulates flagellar motility in pathogenic *Vibrio harveyi. Environ. Microbiol.* **2015,** *17*, 960-968.
(26) Yang, Q.; Pande, G.S.J.; Wang, Z.; Lin, B.; Rubin, R.A.; Vora, G.J.; Defoirdt, T. Indole signaling and (micro)algal auxins decrease the virulence of *Vibrio campbellii*, a major pathogen of aquatic organisms. *Environ. Microbiol*. **2017,** *19*, 1987-2004.
(27) Harris, L.; Owens, L.; Smith, S. A selective and differential medium for *Vibrio harveyi. Appl. Environ. Microbiol.* **1996,** *62*, 3548-3550.
(28) Beuchat, L.R. Interacting effects of pH, temperature, and salt concentration on growth and survival of *Vibrio parahaemolyticus. Appl. Microbiol.* **1973**, *25,* 844-846.
(29) Liu, Z.; Zhao, Q.; Wang, K.; Lee, D.; Qiu, W.; Wang, J. Urea hydrolysis and recovery of nitrogen and phosphorus as MAP from stale human urine. *J. Environ. Sci.* **2008,** *20*, 1018-1024.
(30) Defoirdt, T. Implications of ecological niche differentiation in marine bacteria for microbial management in aquaculture to prevent bacteial disease. *PLOS Path*. **2016,** *12*, e1005843.
(31) McConnelly, J.M.; Jackson, B.; Rothman, A.P.; Klapper, I.; Gerlach, R. Estimation of a biofilm-specific reaction rate: kinetics of bacterial urea hydrolysis in a biofilm. *npj Biofilms Microbiomes* **2015***, 1*, 15014.
(32) Allison, C.; Lai, C.H.; Hughes, C. Co-ordinate expression of virulence genes during swarm cell differentiation and population migration of *Proteus mirabilis. Mol. Microbiol*. **1992,** *6*, 1583-1591.

TABLES

Table 1: *Vibrio campbellii* and *Vibrio harveyi* strains used in this study.

Species	Strain	Reference
Vibrio campbellii	LMG 21361 (= CAIM 415)	17
Vibrio campbellii	LMG 21362 (= CAIM 333)	17
Vibrio campbellii	LMG 21363 (= CAIM 372)	17
Vibrio campbellii	LMG 22888 (= CAIM 416)	17
Vibrio campbellii	LMG 22889 (= CAIM 417)	17
Vibrio campbellii	LMG 22890 (= CAIM 395)	17
Vibrio campbellii	LMG 22895 (= CAIM 223)	17
Vibrio campbellii	BB120 (= ATCC BAA-1116)	17
Vibrio harveyi	LMG 22891	17
Vibrio harveyi	LMG 22893	17
Vibrio harveyi	LMG 22894	17
Vibrio harveyi	E022	18
Vibrio harveyi	VIB 571	19
Vibrio harveyi	VIB 645	19

Table 2: Spearman's correlations between urea degradation (% urea removed) and pH of the medium after 2 days. The calculations were based on the data presented in SI Table 4.

Medium	Correlation ureolysis – pH[1]	Correlation pH - cell density[1]
Undiluted	0.90***	-0.70**
Threefold diluted with water	0.85***	-0.73**
Threefold diluted with urine salts	0.95***	-0.90***
Tenfold diluted with urine salts	NS	NS

[1] Significant correlations are marked with asterisks (**: P < 0.01; ***: P < 0.001; NS: Not significant

Table 3: Ureolysis rates observed in suspensions of strains LMG 22893 and LMG 22895 in the pretreatment experiment. The strains were grown in LB medium, inoculated (10% v/v) in salt solutions with different levels of urea and incubated overnight. The suspensions were centrifuged and resuspended in synthetic urine with 50 mg/l erythromycin to determine ureolytic activity.

Strain	**Ureolysis rate (mg urea L^{-1} h^{-1})**		
	Pretreatment 10 g L^{-1} urea[1]	**Pretreatment 1 g L^{-1} urea[2]**	**Pretreatment 0 g L^{-1} urea[2]**
LMG 22893	3 650	29	0
LMG 22895	4 150	73	10
E022	2 075	ND	ND

[1] Based on the 4h data of Figure 4
[2] Based on the data of SI Figure 5
ND: not determined

FIGURES

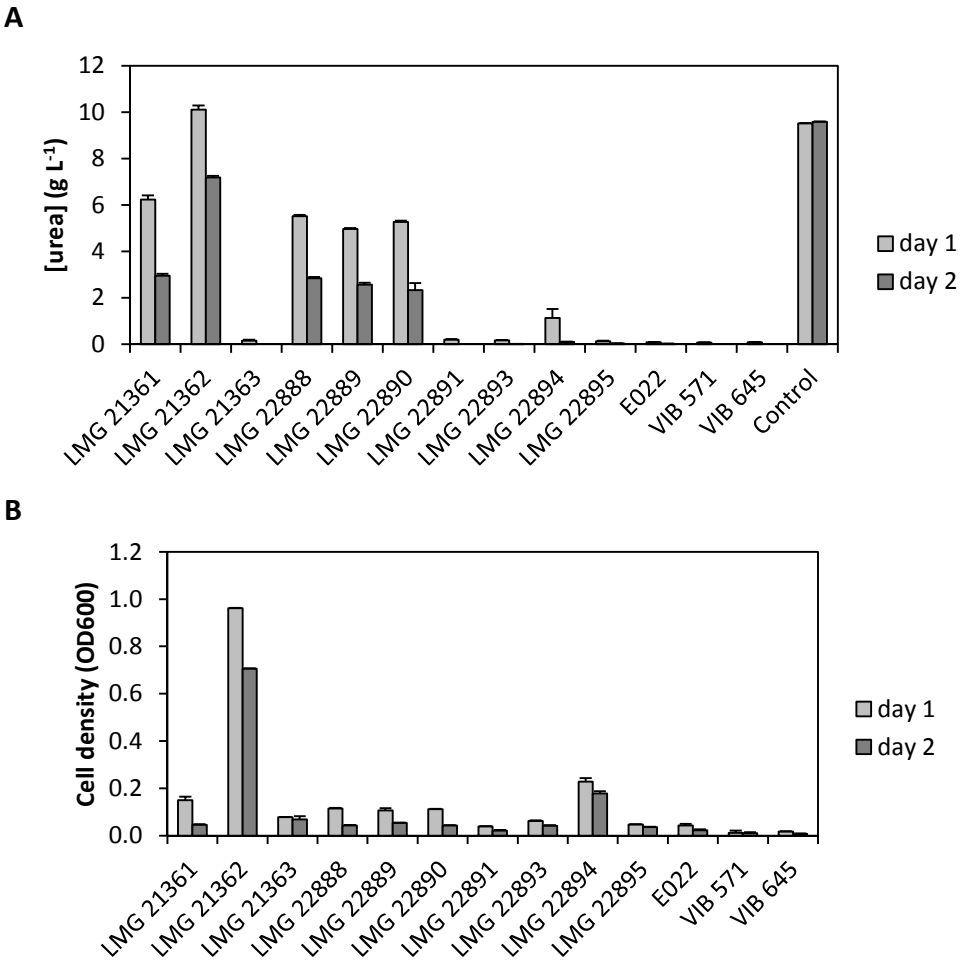


Figure 1: Remaining urea levels (panel A) and cell densities (panel B) of *V. campbellii* and *V. harveyi* cultures in undiluted synthetic urine after 1 and 2 days of incubation at 28°C. Error bars represent the standard deviations of 3 replicate cultures. Sterile synthetic urine was used as a control.

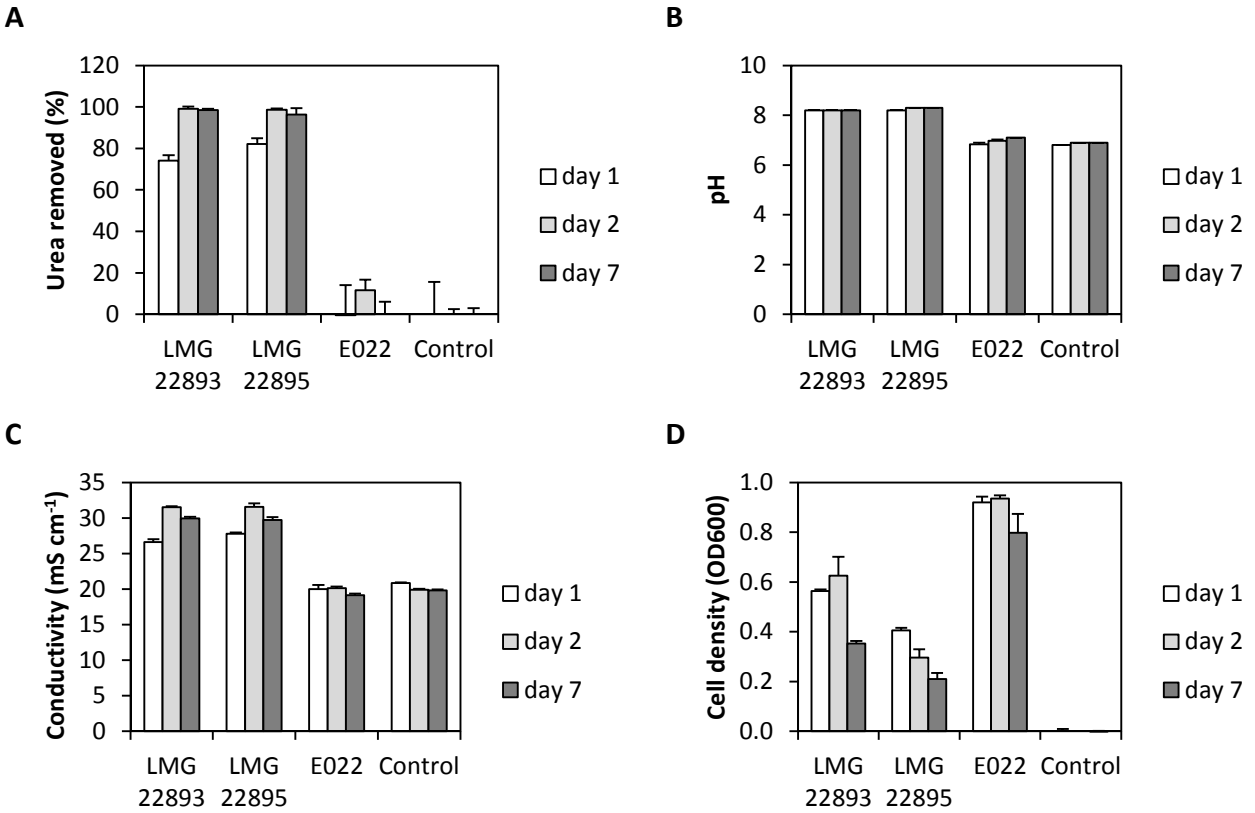


Figure 2: Percentage of urea removed (panel A), pH (panel B), conductivity (panel C) and cell density (panel D) of *Vibrio harveyi* LMG 22893, *Vibrio campbellii* LMG 22895 and *Vibrio harveyi* E022 cultures after 1, 2 and 7 days of incubation at 28°C in synthetic urine that was threefold diluted in phosphate buffer (final concentration 50 mM, pH 7). Error bars represent the standard deviation of 3 replicate cultures. Control refers to sterile medium that was treated in the same way as the *Vibrio* cultures.

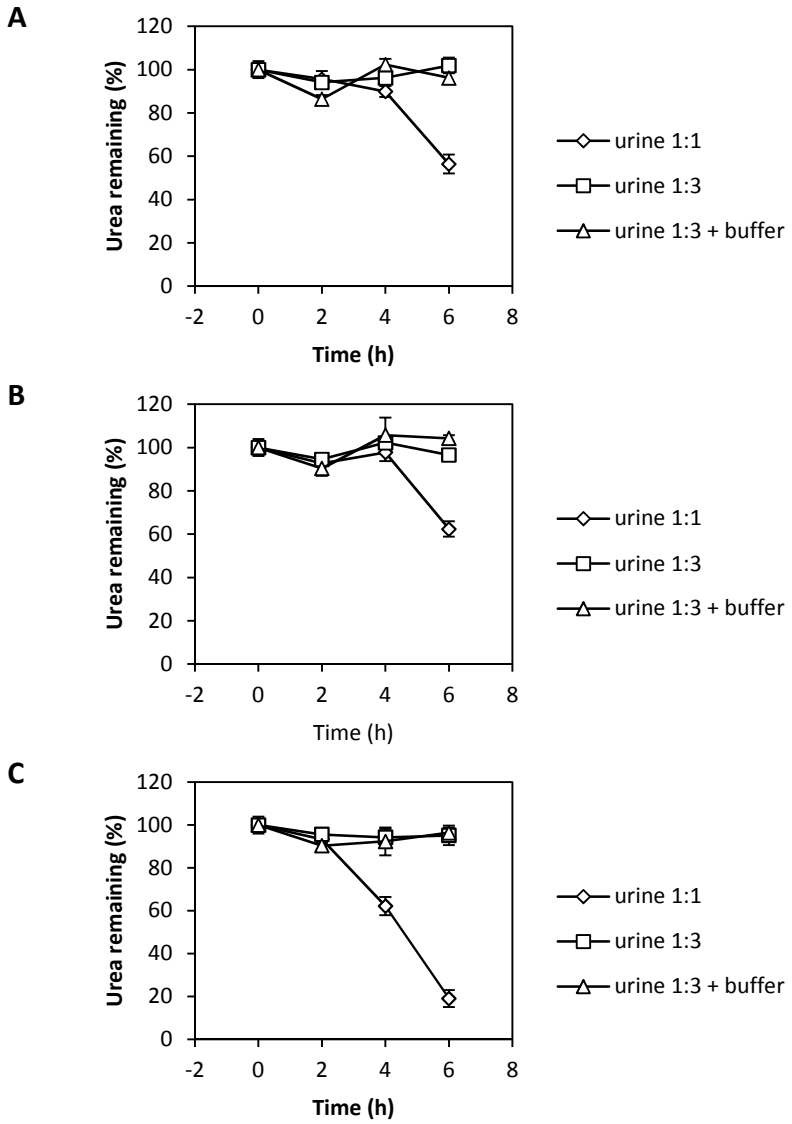


Figure 3: Urea hydrolysis by *Vibrio harveyi* LMG 22893 (panel A), *Vibrio campbellii* LMG 22895 (panel B) and *Vibrio harveyi* E022 (panel C) in undiluted (1:1) and threefold (1:3) diluted synthetic urine (either in distilled water or phosphate buffer (final concentration 50 mM, pH 7)). Error bars represent the standard deviation of 3 replicate cultures.

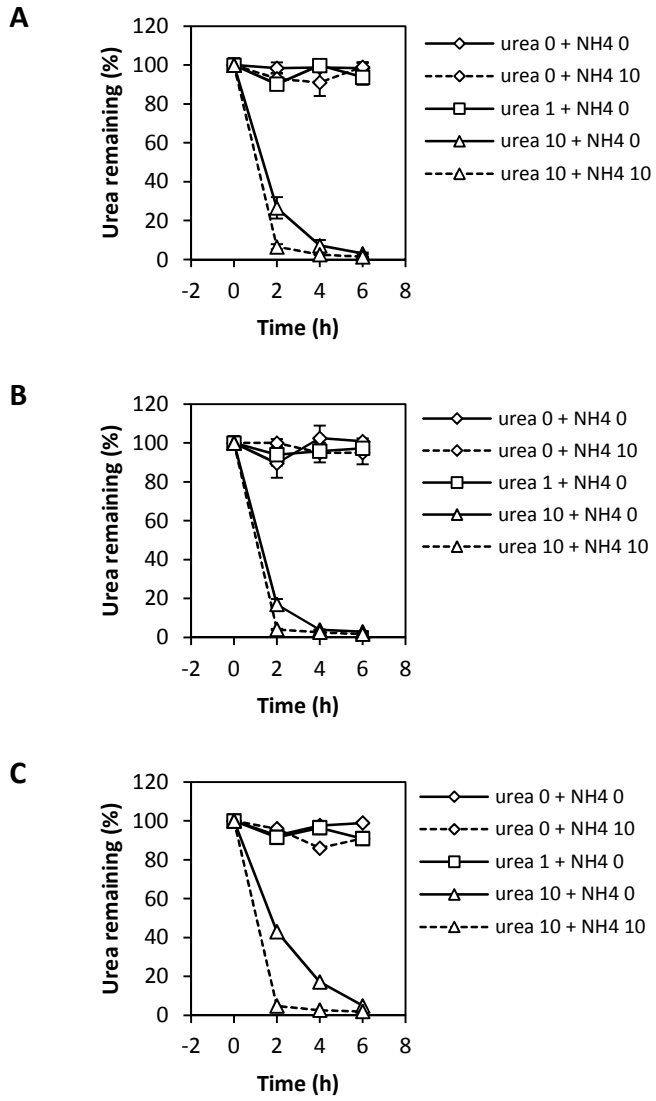


Figure 4: Remaining urea levels in synthetic urine containing pretreated *Vibrio harveyi* LMG 22893 (panel A), *Vibrio campbellii* LMG 22895 (panel B) or *Vibrio harveyi* E022 (panel C). The bacteria were pretreated overnight in salts solutions containing different levels of urea (0, 1 or 10 g L^{-1}), with or without 10 g L^{-1} ammonium. The pretreated cultures were centrifuged, salts solutions were discarded and cells were resuspended in equal volumes of synthetic urine (supplemented with 50 mg L^{-1} erythromycin to inhibit *de novo* synthesis of urease) at the start of the experiment. Error bars represent the standard deviation of 3 replicate cultures.

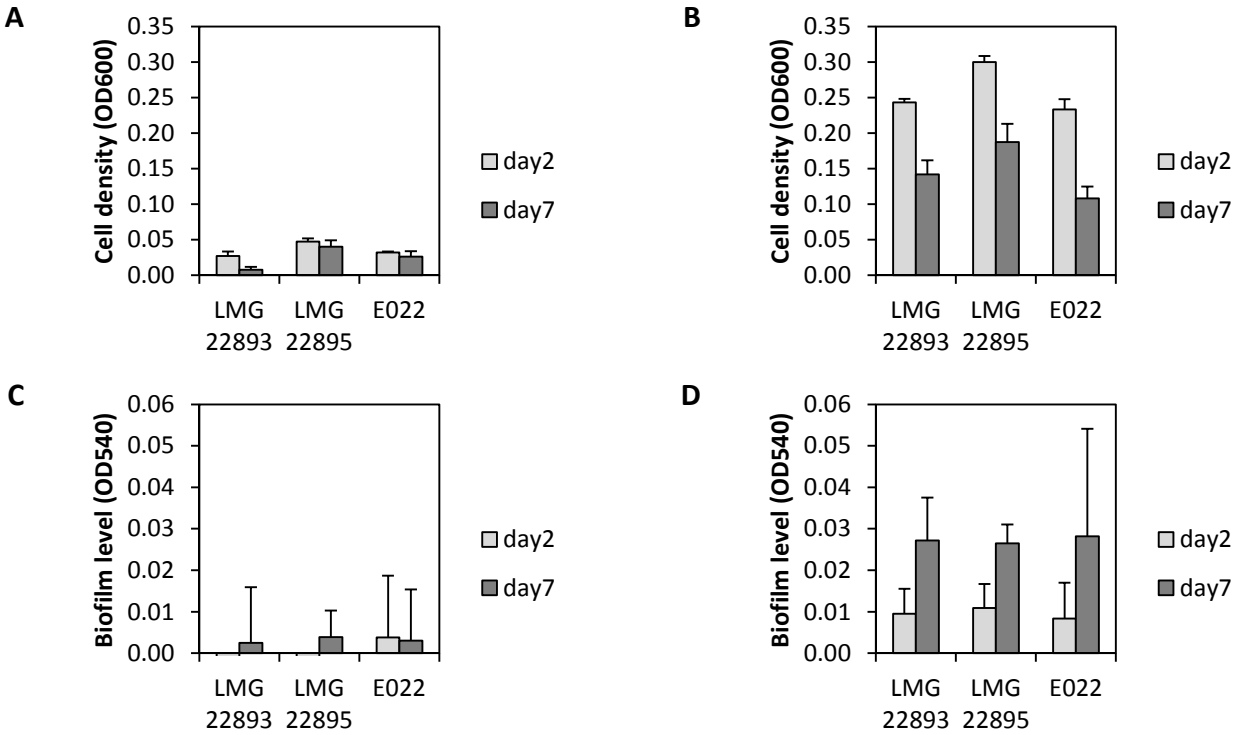


Figure 5: Growth (panels A and B) and biofilm levels (panels C and D) of *Vibrio harveyi* LMG 22893, *Vibrio campbellii* LMG 22895 and *Vibrio harveyi* E022 in the wells of a 96-well polystyrene plate containing either buffered threefold diluted synthetic urine (panels A and C) or LB medium (panels B and D). Error bars represent the standard deviations of 6 replicate cultures.

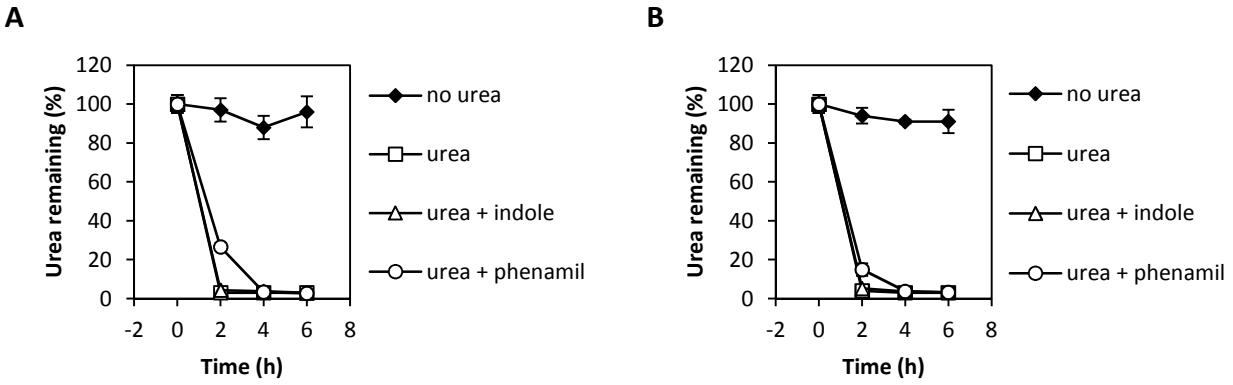


Figure 6: Remaining urea levels in synthetic urine containing pretreated *Vibrio harveyi* LMG 22893 (panel A) or *Vibrio campbellii* LMG 22895 (panel B). The bacteria were pretreated overnight in salt solutions containing 10 g L^{-1} urea, with or without 200 µM indole or 50 µM phenamil. The pretreated cultures were centrifuged, salts solutions were discarded and cells were resuspended in equal volumes of synthetic urine (supplemented with 50 mg L^{-1} erythromycin to inhibit *de novo* synthesis of urease) prior to the experiment. Error bars represent the standard deviation of 3 replicate cultures.