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H-Aquil: a chemically defined cell culture medium for trace metal studies in *Vibrios* and other marine heterotrophic bacteria

Donald E. Martocello III · François M. M. Morel · Darcy L. McRose

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Abstract A variety of trace metals, including prominently iron (Fe) are necessary for marine microorganisms. Chemically defined medium recipes have been used for several decades to study phytoplankton, but similar methods have not been adopted as widely in studies of marine heterotrophic bacteria. Medium recipes for these organisms frequently include tryptone, casamino acids, as well as yeast and animal extracts. These components introduce unknown concentrations of trace elements and organic compounds, complicating metal speciation. Minimal medium recipes utilizing known carbon and nitrogen sources do exist but often have high background trace metal concentrations. Here we present H-Aquil, a version of the phytoplankton medium Aquil adapted for marine heterotrophic bacteria. This medium consists of artificial seawater supplemented with a carbon source, phosphate, amino acids, and vitamins. As in Aquil, trace metals are controlled using the synthetic chelator EDTA. We also address concerns of EDTA toxicity, showing that concentrations up to 100 µM EDTA do not lead to growth defects in the copiotrophic bacterium *Vibrio harveyi* or the oligotrophic bacterium *Candidatus* Pelagibacter ubique HTCC1062, a member of the SAR11 clade. H-Aquil is used successfully to culture species of *Vibrio*, *Phaeobacter*, and *Silicibacter*, as well as several environmental isolates. We report a substantial decrease in growth rate between cultures grown with or without added Fe, making the medium suitable for conducting Fe-limitation studies in a variety of marine heterotrophic bacteria.

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D. E. Martocello III · F. M. M. Morel · D. L. McRose
Department of Geosciences, Princeton University, Princeton, NJ 08544, USA

Present Address:
D. E. Martocello III
Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

Present Address:
D. E. Martocello III
Department of Earth, Atmospheric, and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Present Address:
D. L. McRose (✉)
Divisions of Geological and Planetary Sciences and Biology and Biological Engineering, California Institute of Technology, 1200 E California Blvd., Pasadena, CA 91125, USA
e-mail: dmcrose@caltech.edu

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Introduction

Iron (Fe) is employed in many essential biochemical reactions, including electron transport, nitrogen fixation, and photosynthesis. Extremely low concentrations of bioavailable Fe in much of the surface ocean make it a limiting micronutrient for marine microbial growth (Martin et al. 1990). Studying Fe acquisition in marine heterotrophic bacteria, microorganisms that account for approximately 50% of the oceans' carbon biomass (Fuhrman et al. 1989), requires a defined medium with controlled, low background metal concentrations. Medium recipes for marine heterotrophic bacteria often draw from those used for terrestrial heterotrophic bacteria and utilize hydrolyzed yeast, autolyzed yeast, hydrolyzed casein, animal brain or heart infusions, or some combination thereof [some example of recipes for heterotrophs across environments include: LB (Bertani 1951), Zobell marine broth 2216 (Zobell 1941), nutrient broth (Sigma 70122), terrific broth (Cold Spring Harbor Laboratories 2010b), and King's B medium (King et al. 1954)]. These 'complex' or 'rich' media are very useful for the cultivation of microorganisms. However, the inclusion of undefined components introduces variable background levels of trace metals and greatly complicates their chemical speciation, which depends on the extent of complexation by unknown organic compounds. Minimal medium recipes are available for heterotrophic bacteria [for example, M9 minimal medium (Cold Spring Harbor Laboratories 2010a) and many others (Farmer and Hickman-Brenner 2006; Madigan et al. 2009; Neidhardt et al. 1974)]. However, these medium recipes do not typically control trace metal speciation. And, in some cases, recipes rely on tap water as the metal source, leaving metal concentrations both unknown and variable. As a result, sustained trace metal limitation cannot be achieved because (i) metal levels are simply too high; (ii) unbuffered concentrations of bioavailable metals provide no useful experimental window between metal-replete and deplete conditions; and/or (iii) trace metals precipitate from solution.

The problem of trace metal speciation has been successfully addressed in phytoplankton growth media through the use of fully chemically defined recipes and the addition of synthetic chelators (Brand et al. 1986; Guillard and Ryther 1962; Morel et al. 1979; Provasoli et al. 1956), that complex metals and allow for their slow replenishment in the growth medium, essentially acting as a chemical chemostat. The use of chelators also has the added advantage of scavenging potentially toxic metal contaminants, which can be of particular concern for sensitive marine organisms (Price et al. 1989). Several options exist for metal chelation (for example: citrate, nitrilotriacetic acid, ethylenediamine-*N*-*N*′-diacetic acid) but, ethylenediaminetetraacetic acid (EDTA) has emerged as a particularly common and useful chelator (Hutner et al. 1950; Schwarzenbach and Ackerman 1948; Sunda et al. 2005). Notably, Fe limitation has been achieved in complex media by cleaning the medium using a Chelex column and adding EDTA (Granger and Price 1999; Roe et al. 2011, 2013). We aimed to develop a fully chemically defined EDTA-buffered medium recipe suitable for the growth of marine heterotrophic bacteria. This medium combines the trace element formula used in the phytoplankton growth medium Aquil (Morel et al. 1979; Price et al. 1989; Sunda et al. 2005) with a carbon source, amino acids, basic salts, and vitamins. The resulting medium, H-Aquil is designed to accomplish two goals: (1) reproducible culturing of a variety of bacteria under physiologically optimal and experimentally useful conditions (Carini et al. 2013; Neidhardt et al. 1974) and (2) facilitation of microbial trace metal studies through precise regulation of metal concentrations.

Our initial efforts focused on culturing marine *Vibrios* as they comprise an abundant and diverse genus of aquatic heterotrophic bacteria that are frequently used in laboratory studies. Glycerol is known to be a viable carbon substrate for many *Vibrios* (Farmer and Hickman-Brenner 2006). Experiments were conducted to optimize the nitrogen source and to reduce the formation of precipitates during medium preparation at oceanic pH (8.1). In addition, we conducted experiments to address the potential toxicity of EDTA. Despite its utility, EDTA is not widely used in studies of heterotrophic bacteria, in part due to concerns over cellular toxicity. At high concentrations, EDTA has been shown to increase membrane permeability, with the practical outcome of

enhancing sensitivity to antibiotics [reviewed in (Hancock 1984; Nikaido 2003; Nikaido and Vaara 1985)]. The goal of much of this previous work was in fact to increase membrane permeability. Accordingly, these studies typically employ EDTA concentrations > 200 μM (and often as high as millimolar) to ensure that this occurs. As a part of recipe development, we assessed the effect of lower concentrations of EDTA on *V. harveyi* growth and demonstrated that the oligotrophic bacterium *Candidatus* Pelagibacter ubique (herein SAR11) can be successfully grown in fully synthetic media [previously developed by (Carini et al. 2013)] with Aquil trace metals containing 100 μM EDTA. Further Fe-limitation experiments with a variety of heterotrophic bacteria demonstrate the utility of H-Aquil for metal-limitation studies in marine heterotrophic bacteria.

Materials and methods

Bacterial cultures

Bacterial strains and cultures were either purchased or generously provided from other laboratories. *Vibrio harveyi BB120* (ATCC BAA-1116) was obtained from the Bassler Lab (Princeton University). This organism was recently re-classified as *V. campbellii* (Lin et al. 2010) but is referred to herein as *V. harveyi* for consistency with previous publications. Strains of *Vibrio anguillarum*, *Vibrio fischeri*, *Phaeobacter inhibens*, and *Silicibacter pomeroyi* were obtained from the Seyedsayamdost Lab (Princeton University). Environmental *Trichodesmium* epibionts (denoted EPI) were gifts from the Dyhrman Lab (Columbia University). *Trichodesmium* colonies from the South Pacific and the North Pacific Oceans were collected by net tow, as previously described (Frischkorn et al. 2017). Bacterial epibiont isolates (South Pacific: EPI-3, EPI-7, and EPI-11; North Pacific: EPI-1B1) were obtained by using a sterile disposable inoculating loop to streak *Trichodesmium* colonies on Difco Marine Agar plates, which were prepared according the manufacturer's instructions. Full descriptions of these organisms are forthcoming (Baars et al. in prep). *Ca.* P. ubique HTCC1062 was obtained from the Giovannoni Lab (High Throughput Microbial Cultivation Lab, Oregon State University). Other *Vibrio* strains were purchased from DSMZ (Leibniz Institute).

Medium preparation and growth experiments

H-Aquil was assembled in acid-cleaned (10% v/v hydrochloric acid, HCl) polypropylene plasticware (Nalgene) from sterile stock solutions of each component (Table 1). All stocks were prepared in polycarbonate 15 or 50 mL tubes with Milli-Q water. After assembly, medium pH was adjusted to 8.1 using HCl and then filter-sterilized using bottle top filters (Stericup 0.22 μm, Millipore). AMS1 was prepared as described by Carini et al. (2013). However, filter sterilization was used instead of autoclaving and the CO_2 and air sparging steps were omitted.

Table 1 The H-Aquil recipe described here is assembled in the order shown

Chemical	Final concentration (M)
Artificial seawater	
NaCl	3.00×10^{-1}
$CaCl_2 \cdot 2H_2O$	1.05×10^{-2}
$MgSO_4 \cdot 7H_2O$	5.00×10^{-2}
H_3BO_3	4.85×10^{-4}
Macronutrients	
Glycerol	6.52×10^{-2}
K_2HPO_4	4.00×10^{-4}
MEM essential amino acids[a]	1.37×10^{-2}
MEM nonessential amino acids[a]	2.80×10^{-3}
Vitamins	
Thiamine	2.90×10^{-6}
Riboflavin	2.70×10^{-8}
Aquil trace metals	
$FeCl_3 \cdot 6H_2O$	1.00×10^{-6}
$MnCl_2 \cdot 4H_2O$	1.21×10^{-7}
$CuSO_4 \cdot 5H_2O$	1.96×10^{-8}
$CoCl_2 \cdot 4H_2O$	5.03×10^{-8}
$Na_2MoO_4 \cdot 2H_2O$	1.00×10^{-7}
Na_2SeO_3	1.00×10^{-8}
$ZnSO_4 \cdot 7H_2O$	7.97×10^{-8}
Na_2EDTA	1.00×10^{-4}

Nutrient and vitamin additions were adapted from (Greenberg et al. 1979) and Aquil trace metals from (Sunda et al. 2005)

[a]Corresponds to total amino acid concentration; full composition and N contributions are listed in Table S2

With the exception of SAR11, all cultures were streaked from freezer stocks onto either LB, Marine Broth 2216, or 1/2 YTSS agar plates (Table S1). Following overnight growth on plates, a single colony was then transferred into 15 mL of experimental medium and cultures were acclimated overnight (~ 16 h). At the start of a growth experiment, cells were inoculated at an OD of 0.01 (600 nm). All experiments were conducted with biological duplicates that were shaken at 200 RPM and grown in polystyrene flasks (Nunc flasks, Thermo Scientific) or 96-well microplates (Corning) at the optimal temperature recommended by DSMZ (Table S1). Growth was tracked by measuring the optical density of cultures (600 nm) with a UV–Visible spectrophotometer (Thermo Fisher Evolution 220) or a microplate reader equipped with a spectrophotometer (Biotek Synergy HTX).

Fe- and Cu-replete experiments utilized 1 μM Fe or 19 nM Cu buffered with 100 μM EDTA. For Fe- and Cu-limitation experiments, Fe or Cu was omitted and H-Aquil was prepared with Sigma BioXtra grade salts (higher purity than ACS grade) to minimize trace metal contamination. Based on ICP-MS measurements, the upper estimate of background Fe in H-Aquil prepared with ACS grade salts is 100 nM. For experiments with Sargasso Seawater, all salts were omitted and only glycerol, amino acids, phosphate, trace metals, and vitamins were added to the natural seawater base. For cell-transfer experiments, *Vibrio harveyi* cultures were transferred to fresh media every 24 h. A total of three transfers were conducted, allowing for at least 30 generations in metal replete treatments and Cu-deplete conditions and at least 20 generations in Fe-deplete treatments. Unchelated Fe (Fe') concentrations were calculated using Mineql (Westall et al. 1976) ignoring potential complexation by amino acids and assuming a background Fe concentration of 100 nM (measured upper limit).

Growth of SAR11

SAR11 was maintained in AMS1. Before the start of experiments, cells were grown to mid-exponential phase and inoculated into various medium treatments. These included: (1) standard AMS1, (2) AMS1 with Aquil trace metals, (3) AMS1 with Aquil trace metals without Fe, and (4) H-Aquil. Cultures were shaken at 60 RPM and grown in the dark at 20 °C. Growth was tracked for 34 days by flow cytometry (Accuri C6, BD). Briefly, 500 μL of SAR11 culture was preserved using glutaraldehyde (1% final concentration) and then stored at − 80 °C for future analysis. Samples were thawed and stained with SYBR green nucleic acid stain (final dilution 1:10,000) for 15 min immediately before analysis. Calibration standards—BD TruCount Control beads (1 μm) as well as BD Spherotech 8-Peak and 6-Peak Validation beads—were run daily and Fluoresbrite 0.75 μm YG beads (Polysciences) were used as internal standards in all samples.

Results

Development and optimization of H-Aquil nutrients

Vibrio harveyi was chosen as a model organism due to its fast growth rate, sequenced genome, and genetic tractability. Two common media for culturing *V. harveyi* are L-marine [a version of LB with added salt: 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 20 g L^{-1} NaCl, as described in (Bassler et al. 1994)] and the Autoinducer Bioassay (AB) medium described in (Greenberg et al. 1979). Following the AB recipe, we used glycerol as a carbon source. To determine an optimal defined nitrogen source, we cultured *V. harveyi* in H-Aquil medium (Table 1) prepared with NH_4Cl, $NaNO_3$, or amino acids. Amino acids were added in the form of minimal essential medium (MEM) essential and nonessential amino acid liquid stock solutions [EAA and NEAA, respectively, (Eagle 1955], Table S2). These defined mixtures have been utilized for culturing a broad variety of cells and are commercially available, allowing for efficient medium preparation. We determined that 28 mM N was optimal as lowering total N led to a reduction in growth rate while increasing it had a negligible effect on growth (Table 2). Unsurprisingly, we found that cultures had higher growth rates when provided with amino acids rather than equivalent concentrations of N supplied as NH_4Cl or $NaNO_3$. The omission of suites of amino acids is possible, but leads to slower growth rates (Table 2).

Two other aspects of the medium also required optimization: phosphate concentrations and vitamin additions. AB medium utilizes high (1 mM) levels of

Table 2 Summary of *V. harveyi* growth rates in variations of H-Aquil

Treatment	Nitrogen/Phosphate/pH/Fe_T/Fe′	Growth rate
Standard H-Aquil	28 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.69 ± 0.02
Nitrogen manipulations		
+ 2xEAA + 2xNEAA	56 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.68 ± 0.02
+ ½xEAA + ½xNEAA	14 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.58 ± 0.02
+ EAA + NH_4Cl	28 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.51 ± 0.01
+ NEAA + NH_4Cl	28 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.29 ± 0.02
+ NH_4Cl	28 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.24 ± 0.02
+ $NaNO_3$	28 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.14 ± 0.02
Phosphate manipulations		
1 mM K_2HPO_4[a]	28 mM/1 mM/8.1/1.1 μM/1.7 nM	0.65 ± 0.03
1 mM K_2HPO_4	28 mM/1 mM/7.8/1.1 μM/1.7 nM	0.65 ± 0.02
0.25 mM K_2HPO_4	28 mM/0.25 mM/8.1/1.1 μM/1.7 nM	0.64 ± 0.01
0.10 mM K_2HPO_4	28 mM/0.1 mM/8.1/1.1 μM/1.7 nM	0.49 ± 0.01
Vitamin manipulations		
− Thiamine	28 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.66 ± 0.02
− Riboflavin	28 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.65 ± 0.01
− Thiamine and riboflavin	28 mM/0.4 mM/8.1/1.1 μM/1.7 nM	0.68 ± 0.02
EDTA manipulations		
75% Aquil trace metals	28 mM/0.4 mM/8.1/850 nM/1.8 nM	0.65 ± 0.01
50% Aquil trace metals	28 mM/0.4 mM/8.1/600 nM/1.9 nM	0.65 ± 0.02
25% Aquil trace metals	28 mM/0.4 mM/8.1/350 nM/2.2 nM	0.65 ± 0.01

Growth rates are reported with the standard deviation of biological duplicates

Fe′ denotes the sum of all bioavailable inorganic Fe species and shows slight variation due to the contribution of background Fe (100 nM, see "Methods" section)

[a]Precipitation was observed in this treatment. *NEAA* non-essential amino acids, *EAA* essential amino acids. The full composition for NEAA and EAA are listed in Table S2

K_2HPO_4. However, at oceanic pH in our H-Aquil medium, these K_2HPO_4 additions led to the formation of precipitates. A final concentration of 0.4 mM K_2HPO_4 was determined to yield optimal growth without precipitation at pH 8.1 (Table 2). The original formulation of the AB medium also includes thiamine and riboflavin. In our studies, *V. harveyi* growth in H-Aquil was not substantially affected by the presence or absence of riboflavin and thiamine (Table 2) and searches of the genome suggest that the organism has all genes needed for the biosynthesis of these vitamins (data not shown). However, the riboflavin and thiamine additions from AB medium were still duplicated in H-Aquil as they may be useful additions for other heterotrophic bacteria.

After taking these steps to optimize the medium, we assessed the utility of H-Aquil as compared to established AB and L-marine media. L-marine (which contains NaCl, tryptone, and yeast extract) yielded the fastest growth rates (0.84 ± 0.01). However, growth in H-Aquil (0.69 ± 0.02) was comparable to that in the AB medium (0.73 ± 0.02) and allowed for the completion of a growth cycle (exponential through stationary) within 8 h; an experimentally tractable growth rate (Fig. 1a).

Tests of EDTA toxicity in copiotrophic and oligotrophic marine bacteria

Although EDTA has been successfully utilized as a chelator in numerous phytoplankton studies (Brand et al. 1986; Guillard and Ryther 1962; Morel et al. 1979; Provasoli et al. 1956), it has been less frequently adopted in studies of marine bacteria. In some cases, the omission of EDTA is the result of following established recipes without this (or any other) chelator. However, there is also some concern about EDTA toxicity. *V. harveyi* achieves robust growth rates in the presence of 100 μM EDTA (Fig. 1a). However, this growth rate could still mask some mild EDTA toxicity—notably, growth rates in H-Aquil were not as high as those in L-marine or AB (Fig. 1a). Direct tests of EDTA toxicity were conducted by lowering the amount of total Aquil trace metals (including EDTA) added to H-Aquil. This experimental manipulation leads to a decrease in both the EDTA and total

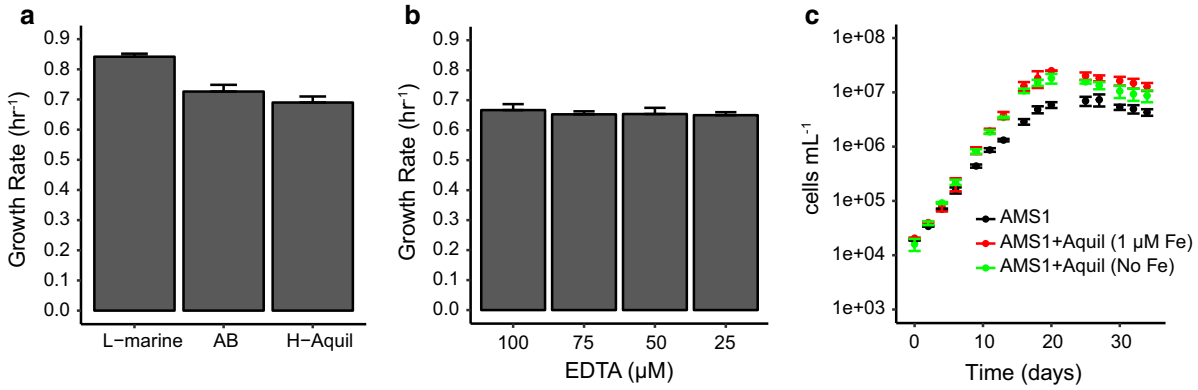

Fig. 1 Growth of *Vibrio harveyi* and SAR11. **a** Growth of *V. harveyi* in L-marine, Autoinducer Bioassay (AB), and H-Aquil medium. **b** Growth rates for *Vibrio harveyi* cultured with varying amounts of EDTA. EDTA and total Fe (Fe_T) are lowered concomitantly and the available Fe (Fe′, the Fe that is not chelated by EDTA) remains roughly the same, with only minor differences due to background Fe. **c** Growth of SAR11 in AMS1 (black) and AMS1 supplemented with Fe-replete (red) and Fe-deplete (green) Aquil trace metals buffered with 100 μM EDTA. Reported growth rates and cell mL^{-1} represent the average for biological duplicates

Fe (Fe_T) concentrations and does not significantly affect the unchelated iron (Fe′), and hence Fe availability. These experiments showed that growth of *V. harveyi* cultures with 25, 50, 75 and 100 μM EDTA was very similar, with treatments with 100 μM EDTA actually showing slightly although not significantly higher growth rates than those with 25 μM EDTA (0.67 vs. 0.65 h^{-1}, Fig. 1b).

We also explored EDTA toxicity in SAR11, an oligotrophic organism with very specific organic requirements. A fully defined growth medium, AMS1 has recently been published for SAR11 (Carini et al. 2013) but does not include EDTA due to concerns over toxicity. We cultured SAR11 in standard AMS1 (no added EDTA and 1 μM Fe) and AMS1 substituted with Aquil trace metals (100 μM EDTA and 1 μM Fe). In addition to the omission of EDTA, the AMS1 and Aquil trace metal components differ in their concentrations of Co, Mn, Mo, Se and Zn. Aquil also contains Cu, which is absent from AMS1, but no Ni, which is present in AMS1. SAR11 grew slightly faster in AMS1 substituted with Aquil trace metals (with EDTA) than in AMS1 ($\mu = 0.44 \pm 0.03$ vs 0.36 ± 0.01) and achieved a higher maximum cell concentration (Fig. 1c). Attempts to grow this organism in H-Aquil were unsuccessful (Fig. S1). We observed no difference in growth rate between SAR11 cultures grown in AMS1 amended with Aquil trace metals containing Fe and those in which Fe was omitted. However, cell yields were slightly higher in treatments with added Fe than those without (Fig. 1c).

Growth and Fe-limitation of *Vibrios* and other marine heterotrophic bacteria in H-Aquil

We further explored the ability of H-Aquil to support the growth of several *Vibrio* species as well as other marine heterotrophic bacteria. *Vibrio* strains were selected to capture the diversity of this genus (Sawabe et al. 2013; Thompson et al. 2007). Other frequently studied marine heterotrophic bacteria from multiple genera as well as environmental epibionts isolated from *Trichodesmium* colony surfaces were also cultured. All species tested grew in standard H-Aquil containing 1 μM Fe (Figs. 2a, b, S2). However, absolute growth rates varied greatly between different bacteria and while most species entered exponential phase immediately after inoculation, lag phases were sometimes observed (Fig. S2). We grew several species of heterotrophic marine bacteria in H-Aquil with no Fe added; observing an average decrease in growth rate between Fe-replete and Fe-deplete cultures of 42 ± 12% (standard deviation, Figs. 2b, S2).

Experiments were also conducted to explore the stability of Fe-limited growth rates. Cultures were transferred multiple times in Fe-deplete medium to exhaust cellular metal stores. As expected, growth was slower in Fe-deplete as opposed to Fe-replete cultures. However, in general, growth rates for Fe-replete and

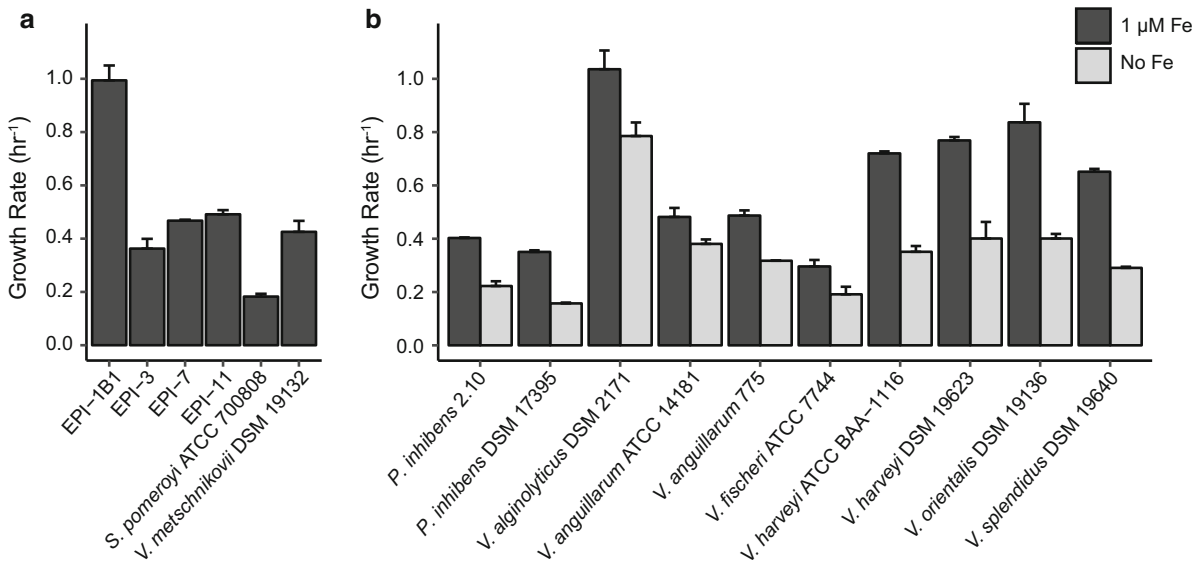

Fig. 2 Growth and Fe limitation of marine heterotrophic bacteria in H-Aquil. **a** Growth of environmental isolates obtained from the surface of *Trichodesmium* colonies and other heterotrophs in Fe-replete (1 μM Fe_T) H-Aquil. **b** Growth and Fe limitation of marine heterotrophs in H-Aquil with 1 μM Fe_T and no added Fe

deplete cultures were constant across transfers, suggesting that cellular reserves were sufficiently exhausted by overnight acclimation in Fe-deplete medium (Fig. 3). Notably, similar experiments conducted with Sargasso seawater (which contains extremely low concentrations of Fe) did not differ from fully synthetic media (Figs. S3, S4). We also explored growth in a medium without added copper (Cu), another biologically essential trace metal. A consistent lag phase was observed in Cu-deplete cultures. However, Cu-replete and deplete cultures had very similar growth rates and achieved similar final cell yields (Fig. S3), possibly due to background Cu levels sufficient for growth. As such, Cu-limitation requires further optimization, but may be an interesting area for future study in this organism.

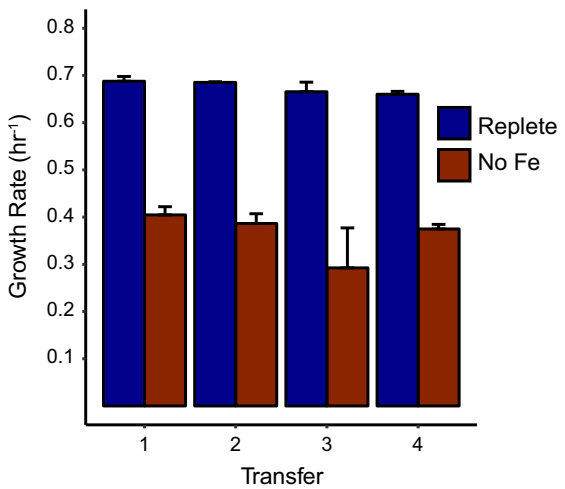

Fig. 3 Stability of *V. harveyi* growth rates in Fe-replete and deplete H-Aquil. Cells were transferred every 24 h to exhaust cellular stores of trace metals. Stable growth rates across transfers suggest that these reserves are negligible. Experiments were conducted in biological duplicates

Discussion

General utility of H-Aquil

In developing H-Aquil, we aimed to produce a chemically defined medium that could be used to conduct trace metal manipulations in a variety of marine heterotrophic bacteria. As our results show, species from numerous genera of marine bacteria can be cultured in H-Aquil, and Fe-limited growth could be achieved for many tested strains. Our results also suggest that high purity salts do not contribute an inordinate amount of metal contamination as growth rates in Sargasso Seawater and synthetic medium were not appreciably different (Fig. S4). Notably, our experiments were conducted with easily accessible

plastics (acid-cleaned polycarbonate and polypropylene) that can be adopted in most laboratories. However, if extremely low Fe concentrations are required, more rigorous measures including the use of Chelex to remove Fe from the medium, storage and preparation of solutions in Teflon, and preparation of media in a clean room are recommended [as detailed in (Price et al. 1989; Sunda et al. 2005)]. Such precautions may allow for Fe-limitation in cultures of *Trichodesmium* epibionts and SAR11 (Figs. 1, S1, S2).

For conducting future experiments with H-Aquil, it may be necessary to make slight revisions to allow for the optimal growth of other heterotrophic bacteria, with the most obvious adjustment being utilizing a different carbon source. Added vitamins may also be needed to meet specific organism's requirements. Such modifications are likely necessary for culturing SAR11 in H-Aquil, which lacks known SAR11 growth requirements such as pyruvate and numerous vitamins. In addition, the high nutrient concentrations of H-Aquil may be toxic for SAR11 and other pelagic bacteria.

Use of EDTA for trace metal studies in marine heterotrophic bacteria

A secondary goal of our study was to assess the toxicity of EDTA to heterotrophic bacteria. Our data show that *V. harveyi* growth rates did not vary significantly across treatments that contained 25, 50, 75 and 100 µM EDTA (Fig. 1b), suggesting that 100 µM EDTA is not toxic to this organism. SAR11 actually grew slightly better in AMS1 medium supplemented with Aquil trace metals containing 100 µM EDTA than in standard AMS1 medium (Figs. 1c, S1). The replacement of AMS1 trace metals with Aquil trace metals entails altering some trace metal concentrations and does not provide a perfect comparison. Nonetheless, SAR11 is a notoriously sensitive organism that has been challenging to culture (Carini et al. 2013; Rappé et al. 2002). Its successful growth in the presence of 100 µM EDTA is a positive outcome for use of EDTA in the culturing (and trace metal limitation of) sensitive marine organisms. All of the environmental *Trichodesmium* epibiont isolates tested also grew in the presence of 100 µM EDTA (Fig. 2a).

Notably, some previous work on EDTA suggests that increases in membrane permeability can occur without alterations in growth rate (Leive 1965). Divalent cations such as Ca^{2+} and Mg^{2+} are thought to help stabilize the outer membrane of Gram-negative bacteria by binding negatively charged lipopolysaccharides (Schneck et al. 2010). The mechanism of reported EDTA membrane disruption is the chelation and removal of these cations (Nikaido 2003; Nikaido and Vaara 1985). Several studies (for example, (Brown and Melling 1969; Nicas and Hancock 1983) used EDTA concentrations in the medium greater than those of Ca^{2+} and Mg^{2+} and demonstrated a reduction in cell membrane cation content. But in a seawater-based medium, where cation concentrations greatly exceed those of EDTA (mM for cations vs. µM for EDTA), this is unlikely to be an issue. Indeed, the majority of EDTA in seawater is bound to calcium resulting in a much lower effective affinity of EDTA for trace metals and slower kinetics of trace metal coordination (Hering and Morel 1988; Sunda et al. 2005).

Overall, our Fe-limitation experiments demonstrate that cultures will grow at different rates, depending on the amount of added Fe in H-Aquil, allowing for controlled Fe-limitation experiments. Based on our growth rate data, 100 µM EDTA does not appear toxic for the cultures we tested, although lower levels of this chelator may be needed to culture more sensitive organisms. These results are consistent with other studies that have used complex media containing EDTA to culture marine heterotrophs (Granger and Price 1999; Hopkinson et al. 2008; Roe et al. 2011, 2013). We suggest that EDTA is useful in both general cell culturing and Fe-limitation applications with marine heterotrophic bacteria.

Conclusion

H-Aquil is a fully defined cell culture medium that allows for robust growth and facilitates Fe-limitation studies in a variety of marine heterotrophic bacteria. H-Aquil can be used to grow numerous species of *Vibrio, Phaeobacter*, and *Silicibacter*, as well as environmental epibiont isolates from *Trichodesmium* colony surfaces. Additionally, our experiments show that 100 µM EDTA does not induce growth defects in either *V. harveyi* or SAR11 and suggest that many marine heterotrophic bacteria are likely to successfully grow in the presence of EDTA.

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