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Release and Consumption of D-Amino Acids During Growth of Marine Prokaryotes

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Abstract Analysis of the composition of the marine-dissolved organic matter has highlighted the importance of D-amino acids, whose origin is attributed mainly to the remains of bacterial peptidoglycan released as a result of grazing or viral lysis. However, very few studies have focused on the active release of D-amino acids by bacteria. With this purpose, we measured the concentration of dissolved amino acids in both enantiomeric forms with two levels of complexity: axenic cultures of *Vibrio furnissii* and *Vibrio alginolyticus* and microcosms created from marine microbial assemblages (Biscay Bay, Cantabrian Sea) with and without heterotrophic nanoflagellates (HNFs). Axenic cultures showed that only D-Ala was significantly released and accumulated in the medium up to a concentration of 120 nM, probably as a consequence of the rearrangement of peptidoglycan. The marine microbial assemblages showed that only two D-amino acids significantly accumulated in the environment, D-Ala and D-aspartic acid (Asp), in both the absence and presence of HNFs. The D/L ratio increased during the incubation and reached maximum values of 3.0 to 4.3 for Ala and 0.4 to 10.6 for Asp and correlated with prokaryotic and HNF abundance as well as the rate of prokaryotic thymidine and leucine incorporation. Prokaryotes preferentially consumed L-amino acids, but the relative uptake rates of D-Ala significantly increased in the growth phase. These results demonstrate that bacteria can release and consume D-amino acids at high rates during growth, even in the absence of viruses and grazers, highlighting the importance of bacteria as producers of dissolved organic matter (DOM) in the sea.

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Introduction

The interactions of marine bacteria and archaea with organic matter strongly influence marine biogeochemistry, with important consequences for ocean ecology and global climate change. Dissolved organic matter (DOM) is the dominant form of organic matter in the oceans, and heterotrophic prokaryotes are the main consumers of marine DOM [35]. The prokaryotes transform the utilisable fraction (UDOM) into CO_2 and biomass, and their role as mineralisers and producers of new prokaryotic biomass has been widely studied [2, 10, 45, 46]. The main source of DOM in marine systems is primary production, but additional sources must contribute to some extent [6]. Protist grazing [36, 48] and viral lysis [34, 49] have been considered the main additional mechanisms, and it has been reported that many marine prokaryotes produce copious amounts of capsular exopolymers [9] that can be sources of DOM [17, 50].

During the last few years, special attention has been paid to the release of DOM by healthy prokaryotes, and some studies with microbial assemblages have shown that prokaryotic cells can transform labile DOM into semi-labile or refractory DOM [5, 15, 22, 26, 40]. However, the exact mechanisms responsible for this transformation are not well understood or quantified, and also, the chemical composition of the released organic compounds remains unknown. The chemical characterisation of biomolecules in marine DOM has shown that bacterial peptidoglycan constituents are widely distributed throughout the water column and confirmed that bacteria are an important source of organic matter in the ocean [11, 32, 43, 52]. These peptidoglycan biomolecules accumulate in

seawater and sediments during diagenesis of the DOM [16, 21, 31, 42]; consequently, they are considered semi-labile or refractory because they resist rapid microbial degradation [35]. Among the constituents of peptidoglycan are the D-amino acids.

Amino acids are important components of the marine DOM pool [4]. The L-isomers are the main constituents of proteins and peptides, whereas D-amino acids are predominant in the bacterial peptidoglycans and some other prokaryotic compounds, such as capsules, teichoic acids, lipoproteins, lipopolysaccharides, siderophores and antimicrobial peptides [8, 20, 31]. In addition to bacteria, some algae, fungi, crustaceans and bivalve molluscs produce D-amino acids [1, 29], but their quantitative significance in natural environments has not been determined. Large differences exist in the percentage of D-amino acids in different environments, and comparison among them is difficult since some studies did not correct their data for racemisation that occurs during acid hydrolysis necessary to release amino acids from proteins [19]. However, the percentage of D-amino acids is unexpectedly high with their origin being almost restricted to some prokaryotic components. McCarthy et al. [32] found that D-amino acids comprised 10–30 % of alanine, aspartic, glutamic and serine in the high molecular weight fraction of oceanic DOM from the central Pacific Ocean, the Gulf of Mexico, and the North Sea; Dittmar et al. [11] found that D-enantiomers represented 21 % of total dissolved aspartic acid in riverine waters and 44 % of total dissolved alanine in deep waters of the eastern Arctic Ocean; Perez et al. [43] reported that they represent 21–41 % of total dissolved alanine and 16–55 % of total dissolved aspartic in North Atlantic waters; and Kawasaki and Benner [22] found that D-enantiomers represented >30 % of total dissolved alanine, aspartic, glutamic and serine in the coastal waters of the North Atlantic. The source of D-enantiomers is probably very diverse but is mainly hypothesised to be the bacterial fragments released by protist grazing [43] or viral lysis [34]. However, some studies [22, 28] have shown that some D-amino acids are actively released during bacterial growth, although the significance and the mechanisms of this release remain to be elucidated.

The study of Kawasaki and Benner [22] demonstrated the bacterial release of D-amino acids during growth of freshwater and marine bacterial assemblages in artificial seawater supplemented with glucose and nutrients. Presumably, the active release of D-amino acids by bacteria varies with growth conditions, and more studies addressing this process under different experimental conditions are needed to understand and evaluate their significance in the flux of carbon in the ocean. Following a completely different approach, the study of Lam et al. [28] analysed the production of D-amino acids by axenic cultures of non-marine bacteria growing in organic rich medium containing tryptone and yeast extract and found that different species produce different D-amino acids such as D-Met, D-Leu, D-Val, D-Tyr, D-Thr, D-Phe, D-Ile and D-Ala. In the present study, we combined both approaches to follow the concentration of two dissolved amino acids, alanine and aspartic acid, in both enantiomeric forms, L and D, during prokaryotic growth under different experimental conditions: marine microbial assemblages growing in natural seawater in presence and absence of grazers and axenic cultures of marine bacterial strains in artificial seawater. The aim was to investigate whether the high D-amino acid concentrations in coastal seawater are mainly a consequence of the activity and growth of prokaryotes and also to evaluate whether grazers and viruses play a significant role. The results demonstrate that prokaryotes can actively release D-Ala and D-aspartic acid (Asp) at high rates during growth, even in the absence of viruses and grazers.

Methods

Experiments with Bacterial Strains

Duplicate experiments with axenic cultures of *Vibrio alginolyticus* (CECT 521) and *Vibrio furnissii* (CECT 4203) were carried out using artificial seawater (ASW) with glucose as the only source of carbon and energy. The ASW medium consisted of a basal salt solution buffered with Tris and enriched with KH_2PO_4, NH_4Cl, trace metal solution, Na_2EDTA as a chelator and vitamins [37]. To avoid precipitation during autoclaving and contamination with organic compounds, the medium was sterilised in a microwave [23]. The sterility of the culture medium was verified by incubating the ASW amended with glucose (10 mg l^{-1}), peptone (10 mg l^{-1}) and casamino acids (10 mg l^{-1}) at 20 °C during 1 week, and no turbidity was observed. Glass material, NaCl and KCl were muffled at 450 °C for 4 h. The trace metal and vitamin solutions were sterilised by filtration through 0.2-μm polyvinylidene difluoride (PVDF) filters (Teknokroma).

The bacterial strains were first cultured in ASW with glucose at a final concentration of 50 mg l^{-1} for 48 h. Cells were collected by centrifugation (2,500 rpm, 15 min), washed twice with ASW and inoculated in 3-l glass flasks with 1 l of ASW supplemented with glucose at 10 mg l^{-1}. Cultures were maintained for 67–70 h in the dark at 20 °C and 100 rpm on an orbital shaker. Subsamples were collected at 6–20-h intervals to determine the abundance of bacteria and viruses and the concentration of dissolved free and combined L- and D-alanine and aspartic acid.

Experiments with Natural Microbial Assemblages

Three experiments were conducted with coastal seawater from the Bay of Biscay (43° 26′ 1.77″N, 2° 54′ 2.19″W) in June, October and November 2009. Samples were collected at a depth of 0.5 m in 2.5-l glass bottles pre-cleaned with diluted

hydrochloric acid and muffled at 500 °C and maintained at 4 °C until being processed in the laboratory within 2 h.

Two types of microcosms were created in order to analyse the effect of heterotrophic nanoflagellates (HNFs) on the concentration of D-amino acids: microbial assemblages with HNF and without the natural HNF community. These assemblages did not receive glucose addition. The assemblages with HNF were prepared with seawater filtered through 8-μm polycarbonate filters in order to remove ciliates and zooplankton, avoiding interactions due to ciliates grazing on HNFs. Gravity filtration was applied to the samples to minimise the potential enrichment of samples with dissolved organic carbon (DOC) generated by cell disruption during the filtration procedure. The assemblages without HNF were prepared with seawater gently filtered through 0.8-μm polycarbonate filters to remove HNFs, though filtration is well known to not be completely effective, and some small HNFs can pass through the filter. Thus, these microcosms were called reduced HNF (R-HNF). Filtration through the 0.8-μm filter could also remove attached and large prokaryotes, but we did not find significant differences in the number of prokaryotes in the HNF and R-HNF microcosms at the beginning of the experiments.

A total of 1.5 l of each type of assemblage was transferred to 2-l glass flasks pre-cleaned with diluted hydrochloric acid and muffled at 500 °C and was incubated in the dark at in situ temperature (12–18 °C depending on the experiments) on an orbital shaker at 120 rpm for approximately 150 h. Subsamples were collected at 10–24-h intervals to measure the number of prokaryotic cells, HNFs and viruses, prokaryotic thymidine and leucine incorporation, D and L-alanine and aspartic acid incorporation and the concentration of dissolved total alanine and aspartic acid.

Microbial Counts

Number of prokaryotic cells was quantified by epifluorescence microscopy according to Porter and Feig [44]. Triplicate 10-ml subsamples were fixed with formalin (final concentration of 2 %) and were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (final concentration of 2 μg ml^{-1}). Within 1 day of preservation at 4 °C, the stained samples were filtered through 0.2-μm-pore-size black Millipore polycarbonate filters. The filters were examined under the Nikon Epifluorescence Microscope; 400 to 600 prokaryotes were counted in at least 20 randomly selected fields. For HNF cell counts, triplicate 2 % paraformaldehyde-fixed subsamples (10 ml) were stained with DAPI (final concentration of 2 μg ml^{-1}). The HNFs in 50–100 randomly selected fields were counted in triplicate samples. Viruses were quantified by epifluorescence microscopy according to Noble and Fuhrman [39]. Triplicate subsamples of 2 ml were filtered through 0.02-μm Acrodisc filters (Whatman) placed on a drop of 0.2 % SYBR Green I (Molecular Probes) and stained for 15 min. Filters were rinsed with 0.02 μm filtered distilled water and mounted on a glass slide, and 300 to 600 viruses were counted on each slide.

Uptake Rates of Thymidine, Leucine and Amino Acids

Thymidine and leucine uptake rates were measured in triplicate 5-ml subsamples incubated with 50 nM [methyl-^{3}H]thymidine (77.1 Ci $mmol^{-1}$, Radiochemical Centre) or 40 nM [methyL-^{3}H]leucine (50–60 Ci $mmol^{-1}$) for 1 h at 20 °C and 120 rpm in the dark. The saturating thymidine and leucine concentrations and the appropriate incubation time were determined prior to the experiments. After incubation, the subsamples were chilled on ice, and 5 ml of ice-cold 10 % (*w*/*v*) trichloroacetic acid (TCA) was added to each subsample. The mixtures were kept on ice for 5 min and then filtered through 0.2-μm membrane filters. The filters were rinsed five times with 5 ml ice-cold 5 % (*w*/*v*) TCA and then dried and transferred to scintillation vials with 0.5 ml ethyl acetate. After 20 min, 4 ml of scintillation liquid (Ultima Gold, PerkinElmer) was added to each vial, and the samples were radioassayed in a liquid scintillation counter (Tri-Carb 2900TR, PerkinElmer). TCA-killed controls (2 % final concentration) were processed in a similar manner.

Amino acid uptake rates were measured according to the procedure by Perez et al. [43]. Triplicate subsamples of 5 ml and two formaldehyde-killed controls were incubated with L-[2,3-^{3}H]Asp, D-[2,3-^{3}H]Asp, L-[2,3-^{3}H]Ala and D-[2,3-^{3}H]Ala (L-Asp 34 Ci $mmol^{-1}$ and D-Asp 12.2 Ci $mmol^{-1}$, BCS, Amersham; L-Ala 20.8 Ci $mmol^{-1}$ and D-Ala 10 Ci $mmol^{-1}$, Moravek Biochemicals) at a final concentration of 10 nM and incubated for 0.5 h at the in situ temperature and 120 rpm. The concentration and incubation time were determined in previous experiments. The addition of amino acids can stimulate the bacterial uptake rate; therefore, the amino acids should be added in a very low amount. On the other hand, the concentration of free L- and D-Ala and L- and D-Asp varied from sample to sample and among the incubations from 0.37 to 87 nM. Therefore, we decided to use a combination of the shortest incubation time and the lowest concentration that yielded a significant number of disintegrations per minute. The concentration of 10 nM was chosen because concentrations lower than 10 nM did not yield sufficient dpm during 30 min of incubation. The incubation was completed by adding formaldehyde at a final concentration of 2 %. The subsamples were filtered through 0.22-μm cellulose acetate filters, rinsed three times with distilled water and then dried and transferred to scintillation vials. Uptake rates were measured after rinsing the filters with distilled water and with 0.2 μm filtered seawater, and results did not show significant differences. Four millilitres of scintillation liquid (Ultima Gold, PerkinElmer) was added, and after 12–18 h, the filters

were radioassayed in a liquid scintillation counter (Tri-Carb 2900TR, PerkinElmer). The radioactivity of killed controls (formaldehyde at 2 % final concentration) was subtracted, and amino acid uptake rates were estimated.

Concentration of Dissolved Amino Acids

Data for all amino acids measured in the microcosms are shown in Electronic Supplementary Material 1. Samples were filtered through 0.22-μm PVDF filters (Gelman) to remove the particulate material and were quantified by reverse-phase high-performance liquid chromatography (HPLC) after pre-column derivatisation with *o*-phthaldialdehyde (OPA) and *N*-isobutyryl-L-cysteine (IBLC) as a chiral agent [13]. OPA/IBLC reagents were prepared fresh every 3 days. A total of 4.8 mg ml^{-1} IBLC and 2 mg ml^{-1} OPA were dissolved in 125 ml methanol and diluted with 875 ml 0.5 M boric acid buffer (pH=10). The reagent was stored at 4 °C. A total of 120 μl of sample and 30 μl of OPA/IBLC solution were drawn into the sample loop and mixed in line for 2 min. The OPA and IBLC mixture forms fluorescent derivatives with amino acids. The concentration of total dissolved amino acids was measured after hydrolysis of the samples, and the concentration of free dissolved amino acids in samples did not undergo hydrolysis. The concentration of combined amino acids was estimated as the difference between the total and free amino acid concentrations.

Samples were hydrolysed by adding 2.5 ml of 30 % HCl (final concentration of 6 N) (Suprapur, Merck) and 20 μl of ascorbic acid (100 μM final concentration) to 1.5 ml of sample and, subsequently, by flushing with N_2 for 20 min. The pre-combusted glass ampoules were sealed and kept at 110 °C for 20 h. The hydrolysed samples were neutralised with 1.3 ml of borate buffer and pH adjusted to 8.5–9.0. The separation of individual amino acids was performed using the Waters 2695 Separation Module with automatic autosampler and detected using the Waters 474 Scanning Fluorescence Detector at an excitation wavelength of 330 nm, emission wavelength of 445 nm and gain of 100. To separate individual amino acids, we used reverse-phase columns, Synergi 4 μm MAX-RP 80A (Phenomenex; 4.6 mm internal diameter×150 mm) for total amino acids and Tracer Excel 120 ODS-B 3 μm (Teknokroma; 4.6 mm internal diameter×150 mm) for free amino acids, and a multistep gradient system as described by Fitznar et al. [13] at a flow rate of 0.8 ml min^{-1}. Mobile phases were as follows: A, aqueous solution of 25 nM sodium acetate (pH 7); B, 100 % methanol gradient quality (ROMIL-SpS Super Purity Solvent, Merck); and C, aqueous solution of 25 nM sodium acetate (pH 5.3). All eluents were degassed before use. The amino acid derivatives were eluted with the following tertiary gradient: $T_{0\ min}$ (91 % A, 5 % B, 4 % C), $T_{48\ min}$ (61 % A, 35 % B, 4 % C), $T_{80\ min}$ (3 % A, 59 % B, 38 % C), $T_{85\ min}$ (21 % A, 75 % B, 4 % C) and $T_{90\ min}$ (91 % A, 5 % B, 4 % C). After 90 min, the system returned to the initial conditions, and the column was equilibrated for 5 min. Amino acid enantiomer standards at different concentrations (7.55, 15.1, 31.2, 62.5, 125, 250 and 500 nM) were processed as above and used for calibration. The relative standard deviation of seawater replicated was 1 to 27 %, including day-to-day variability. Asparagine (Asn) was converted to Asp; thus, the data for L- and D-isomers of Asp reported in this study included Asn. The detection limits (S/N=3) for D-Ala, L-Ala, D-Asp and L-Asp were 0.8, 2.7, 1.5 and 1.3 nM respectively, while quantification limits (S/N=10) were 2.7, 8.3, 5.1 and 4.3 nM. Hydrolysis induces the racemisation of amino acids, and the concentrations of amino acids were corrected according to the percentages estimated by Kaiser and Benner [19]: 1.2 % for Ala and 4.4 % for Asp.

Statistical Analysis

The software IBM SPSS Statistics 21 was used for statistical analyses. The relationship of the concentration of D-amino acids with microbial variables was assessed by calculating Spearman's correlation coefficient. Stepwise multiple linear regression analysis was performed on log-transformed data of those variables that did not show collinearity. The effects of the variable on the multiple regression model with a P value of <0.05 were assumed to be significant.

Results

Experiments with Bacterial Strains

Experiments with axenic cultures of *V. furnissii* and *V. alginolyticus* growing in ASW with glucose as the only source of carbon and energy showed similar trends (Fig. 1 shows a typical experiment). The bacterial abundance increased from 10^4 to 10^7 cells ml^{-1}, and virus-like particle (VLP) was not detected along the incubations. During the log phase, the total D-Ala concentration increased significantly, from 0 to 113 nM in the case of *V. alginolyticus* and up to 120 nM in *V. furnissii*, resulting in an accumulation of D-Ala in the medium. Other amino acids were present at very low or undetectable concentrations and did not show a clear pattern of variation. The free D-Ala concentration increased from 0 to maximum values ranging from 22 to 90 nM (range of all the experiments) during the early log phase. In the late log phase and stationary phase, the free D-Ala concentration decreased, but the combined D-Ala concentration increased. At the end of the incubation, 66–97 % of the D-Ala was in the combined form.

Experiments with Natural Microbial Assemblages

The dynamics of abundance of prokaryotes, HNFs and viruses were similar in all the three experiments (Fig. 2). At the

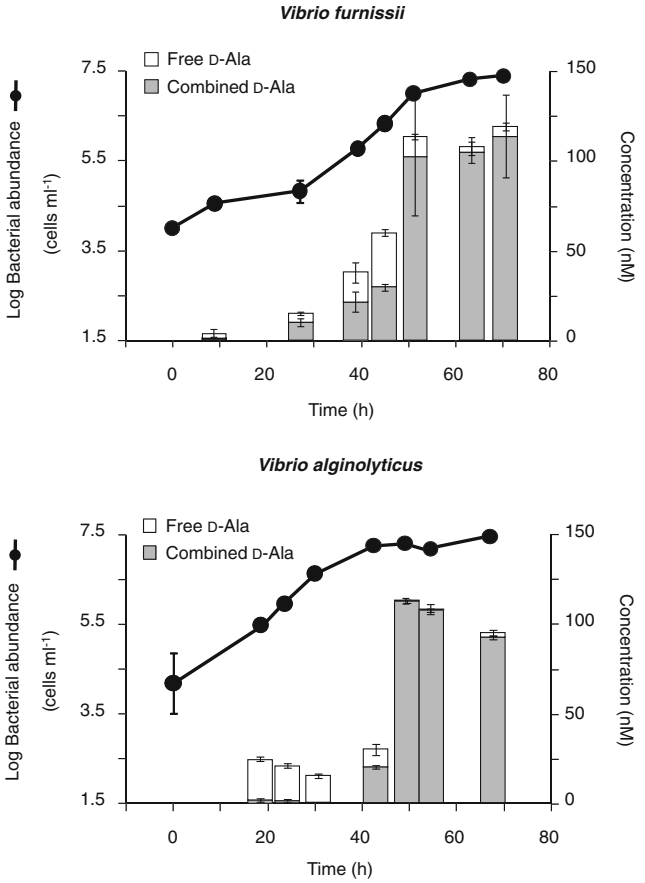

Fig. 1 Time course of the concentration of free and combined D-Ala and the abundance of *V. furnissii* and *V. alginolyticus*. *Bars* represent standard error. If *vertical bars* are not visible, the range is smaller than the size of the *symbol*

beginning of the incubation in the three experiments, the prokaryotic abundance ranged from 0.22×10^6 to 0.46×10^6 cells ml^{-1} in the R-HNF microcosms and from 0.25×10^6 to 0.76×10^6 cells ml^{-1} in the HNF microcosms. The increase in prokaryotic abundance was always smaller in HNF microcosms than R-HNF microcosms (Fig. 2). Across the three experiments, the number of prokaryotes increased during incubation by a factor of 3.2–9.2 in the HNF microcosms and 6.4–15.1 in the R-HNF microcosms. In the R-HNF microcosms, the number of prokaryotes increased during the first 75–115 h, whereas the number of prokaryotes increased during the first 40–60 h in the HNF microcosms (Fig. 2).

As expected, filtration through a 0.8-μm filter to remove HNFs was not completely effective, and some small heterotrophic flagellates grew at the end of the incubation period. However, the number of HNFs was not significant and remained low during the course of the experiments, increasing only at the end (Fig. 2). The period in which the number of HNFs was high in the R-HNF microcosms is represented in the figures by a grey-shaded area (after 100 h of incubation), and data were not included in the statistical analysis. At the beginning of the incubation, the number of HNFs in the HNF microcosms ranged from 196 to 560 HNF ml^{-1}, but they were undetectable in the R-HNF microcosms in the three experiments. The maximum abundance of HNFs in the HNF microcosms ranged from 0.3×10^4 to 2.9×10^4 cell ml^{-1} in the three experiments.

VLP abundance was similar in the HNF and R-HNF microcosms. In the three experiments, the abundance of VLPs ranged from 0.7×10^7 to 3.5×10^7 VLPs ml^{-1}, resulting in an

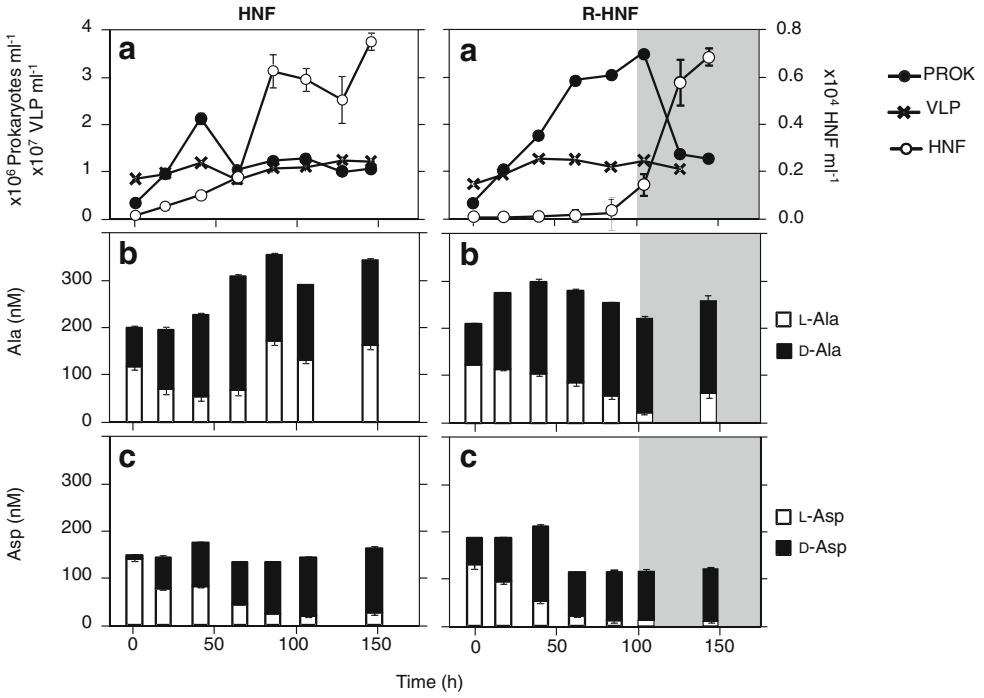

Fig. 2 Time course of microbial abundance (**a**) and concentration of dissolved total L- and D-Ala (**b**) and L- and D-Asp (**c**) during incubation in HNF and R-HNF microcosms. Data from a representative experiment (June) are shown. *Grey-shaded area* indicates the time when the R-HNF microcosms showed a high number of HNFs. *Bars* represent standard error. If *vertical bars* are not visible, the range is smaller than the size of the *symbol*

average virus/prokaryote ratio of 14.4 (range, 3.5–43.0). The highest ratio was found at the beginning of the incubation. Viral abundance increased during prokaryotic growth and then remained constant or declined (Fig. 2).

The composition of amino acids in the surface waters used to create the microcosms was analysed during an annual cycle (data not shown), and alanine and aspartic acid were some of the major amino acids, representing 29.2 % of the total. Among the D-amino acids, D-Ala and D-Asp were predominant. D-Ala represented 27.6 % of the total D-amino acids, whereas the contribution of D-Asp was 12.3 % (Azúa et al. in preparation). Preliminary experiments showed that among D-amino acids, only D-Ala and D-Asp accumulated during the incubations and showed a repetitive pattern of variation. Consequently, in this study, the analysis was restricted to the enantiomeric composition of Ala and Asp, although the amino acid composition in all microcosms is given in ESM 1. The L forms of Ala and Asp were generally found at higher concentrations than the D forms in seawater at the beginning of the incubations (Fig. 2). The D/L ratio ranged from 0.69 to 1.10 for Ala and from 0.06 to 0.41 for Asp (Fig. 3). This ratio showed much greater variability for Asp than for Ala. Nevertheless, the D/L ratio increased during the incubation and reached maximum values of 3.03 to 4.39 for Ala and 0.46 to 9.06 for Asp (Fig. 3). This increase was the consequence of a simultaneous decrease in the concentration of the L forms and increase in the concentration of D forms throughout the incubation (see Fig. 2).

The concentration of D-Ala positively correlated with the abundance of prokaryotes when data from the three experiments were included in the analysis (Spearman rank correlation coefficient $P<0.01$, Table 1). In the HNF microcosms, an increase in the concentration of D-Ala was associated with an increase in the abundance of prokaryotes during the phase of prokaryotic growth (0–50 h), prior to the growth of HNFs (Fig. 2). In the R-HNF microcosms, a significant increase in the D-Ala concentration from the beginning of the incubation was also observed, though this increase was smaller than that of HNF microcosms. The maximum concentration of D-Ala in HNF microcosms was >200 nM, but the concentration in R-HNF microcosms was maintained below this value, although differences were not significant (Fig. 2). The concentration of D-Ala also positively correlated with HNF abundance (Spearman rank correlation coefficient $P<0.01$), but we did not find any correlation with the abundance of viruses. Similarly, the concentration of D-Asp correlated with prokaryote and HNF abundance (Spearman rank correlation coefficient $P<0.05$). The D/L ratio for the concentration of Ala correlated positively with prokaryote and HNF abundance and thymidine and leucine incorporation, and the D/L ratio for Asp correlated with prokaryote and HNF abundance (Table 1). Multiple stepwise regression analysis with log-transformed data revealed that the abundance of prokaryotes was influencing the concentration of D-Ala and D-Asp as well as the ratio D/L-Ala and D/L-Asp (Table 2). The abundance of prokaryotes explained 23 and 30 % of the variation in the concentration of

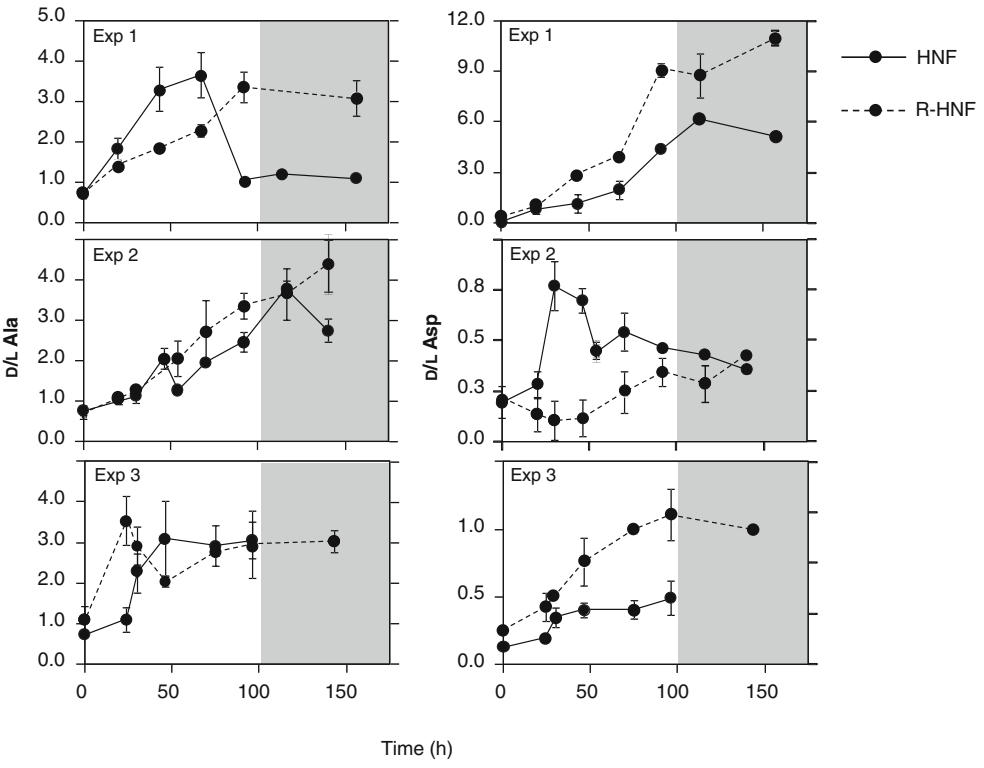

Fig. 3 Time course of the D/L ratio of the concentration of Ala (*left panels*) and Asp (*right panels*) in HNF and R-HNF microcosms in three experiments (*Exp 1*, June 8; *Exp 2*, October 26; and *Exp 3*, November 26). *Grey-shaded area* indicates the time when the R-HNF microcosms showed a high number of HNFs. *Bars* represent standard error. If *vertical bars* are not visible, the range is smaller than the size of the *symbol*

Table 1 Spearman rank correlation coefficients between concentrations of D-Ala, D-Asp, D/L-Ala and D/L-Asp ratios and selected microbial parameters

Parameter	D-Ala	D-Asp	D/L-Ala	D/L-Asp
PROK	0.43**	0.37*	0.59**	0.41**
Tdr	–	–	0.42**	–
Leu	–	–	0.49**	–
HNF	0.51**	0.31*	0.35*	0.33*
VLP	–	–	–	–

Non-significant correlations are not reported

PROK prokaryotic abundance, *Tdr* thymidine incorporation, *Leu* leucine incorporation, *HNF* heterotrophic nanoflagellate abundance, *VLP* virus-like particle abundance

*$P<0.05$; ** $P<0.01$

D-Ala and the ratio D/L-Ala, respectively. The abundance of HNF was excluded from the model ($P>0.05$) in the case of D-Asp concentration and only increased the proportion of the overall variation of D-Ala concentration and D/L-Ala ratio from 23 to 31 % and from 30 to 33 %, respectively.

Relative changes in the thymidine and leucine incorporation rates showed a similar pattern in the three experiments, increasing in the prokaryotic growth phase and decreasing thereafter. The thymidine and leucine uptake rates were lower in HNF microcosms than in R-HNF microcosms (Fig. 4). The uptake rates for Ala were higher than the uptake rates for Asp in all cases ($P<0.05$, Wilcoxon test, Fig. 4). The uptake rates of L- and D-Ala and L- and D-Asp showed similar trends in the HNF and R-HNF microcosms (Fig. 4). The only significant difference was found in the uptake of D-Ala at 20–24 h, which was significantly higher in the HNF microcosms in the three experiments. Generally, the uptake rates for L forms were higher than the uptake rates for D forms (Fig. 4), and this difference was more pronounced in the case of Asp. The D/L ratio for uptake at the beginning of the experiments was low and ranged from 0.16 to 0.58 for Ala and from 0.02 to 0.11 for Asp (Fig. 5). The D/L ratio for the uptake of Asp remained below 0.2 throughout the incubations, but in the case of Ala, it showed an important increase during the prokaryotic growth phase, reaching maximum values in the range of 0.72–1.83 and decreasing at the end of the incubations (Fig. 5).

Table 2 Results of multiple stepwise regression analysis (log-transformed data)

Dependent variable	Independent variables[a]	Adjusted R^2	P
D-Ala	PROK	0.23	0.003
	PROK, HNF	0.31	0.001
D-Asp	PROK	0.16	0.002
D/L-Ala	PROK	0.30	0.002
	PROK, HNF	0.33	0.001
D/L-Asp	PROK	0.18	0.002
	PROK, HNF	0.21	0.001

PROK prokaryotic abundance, *HNF* heterotrophic nanoflagellate abundance, *D-Ala* concentration of D-Ala, *D-Asp* concentration of D-Asp, *D/L-Ala* ratio of the D- and L-Ala concentration, *D/L-Asp* ratio of the D- and L-Asp concentration

[a] Probability to enter, <0.05

Discussion

Our results shed some light on the role of prokaryotes in producing DOM in the sea by showing that an actively growing marine prokaryotic assemblage produces significant amounts of D-amino acids, particularly D-Ala, and that this production is mainly related to the activity of the prokaryotes as they grow. As a first level of complexity, we analysed axenic cultures of *V. alginolyticus* and *V. furnissii* with glucose as the only source of carbon and energy. The results clearly indicate that these two bacterial species release high amounts of D-Ala. The selective release of D-amino acids during cell growth suggests a specific release mechanism involving a particular cellular structure enriched in D-Ala. These results are consistent with those of Kawasaki and Benner [22] who proposed that peptidoglycan synthesis and rearrangement during bacterial growth are responsible for the release of D-amino acids. D-Ala is a component of the peptide side chain of the peptidoglycans in most bacteria. In Gram-negative bacteria, which are the predominant bacteria in the marine environment, the peptide usually consists of L-Ala-D-Glu-(L)-*meso*-diaminopimelic acid (DAP)-D-Ala, but it often lacks D-Ala or, more rarely, terminates in D-Ala-D-Ala [41]. Other amino acids can appear in the peptide chain, but less often in those mentioned here [47]. Cell growth involves the synthesis of peptidoglycan, and the cross links between chains involve transpeptidation. During this reaction, the terminal D-Ala is released by the hydrolysis of D-Ala-D-Ala by a carboxypeptidase, and this reaction of transpeptidation could explain the release of D-Ala during bacterial growth [22].

In the experiments with axenic cultures of *V. furnissii* and *V. alginolyticus*, free D-Ala accumulated in the first half of the exponential phase, and its concentration gradually decreased in the late exponential phase, which suggests that it was reused by the prokaryotes. The synthesis of new peptidoglycan has been reported to involve the release of cell wall peptides, which are recycled for the synthesis of new cell wall material [14]. However, as indicated by Vollmer and Bertsche [55], the peptide composition shows some variation depending on the strain, growth medium and temperature as well as the growth phase. During the transition to stationary phase, the peptidoglycan muropeptide composition changes, and DAP–DAP cross-linking increases, which could involve the release of

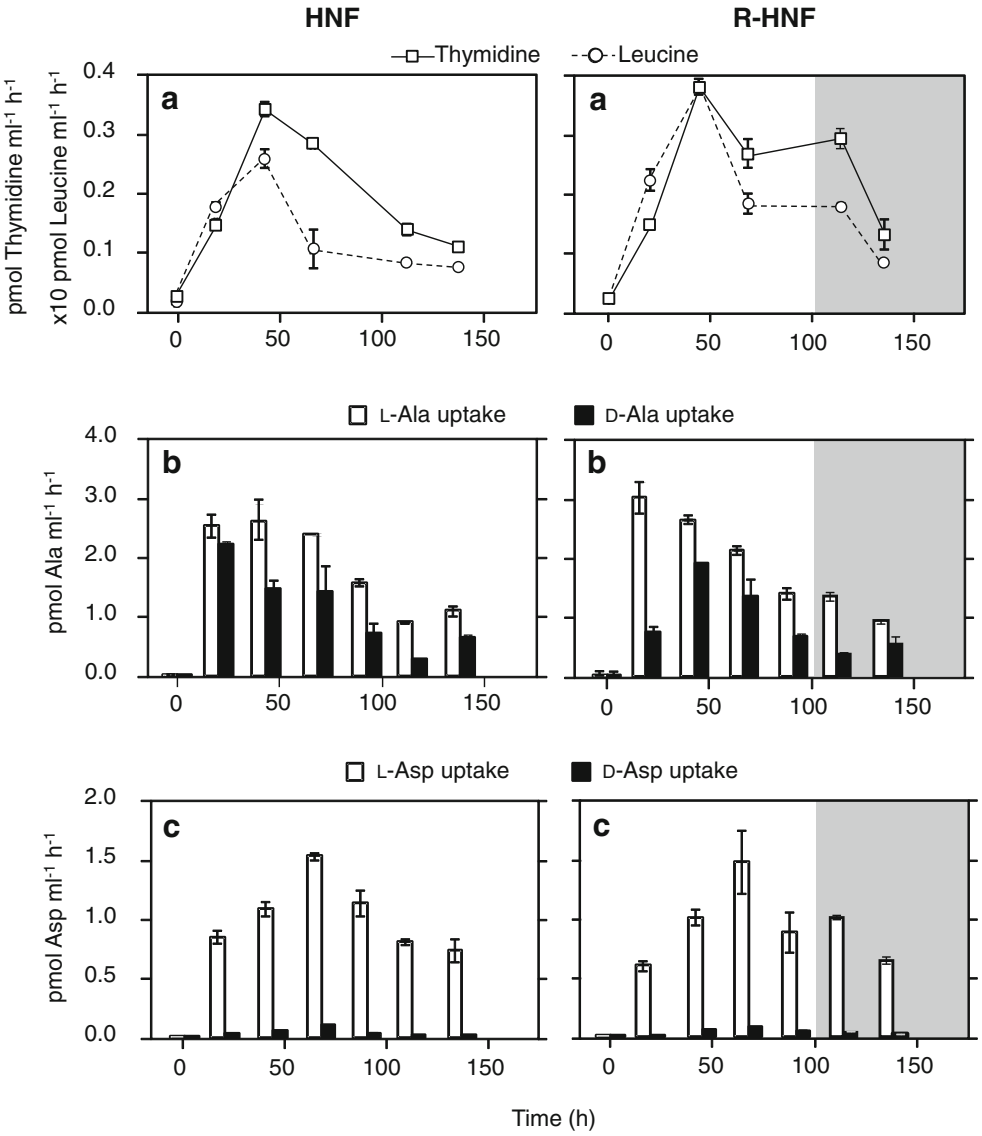

Fig. 4 Time course of thymidine and leucine incorporation (**a**), uptake rates of L- and D-Ala (**b**) and L- and D-Asp (**c**) during incubation in HNF and R-HNF microcosms. Data from a representative experiment (June) are shown. *Grey-shaded area* indicates the time when the R-HNF microcosms showed a high number of HNFs. *Bars* represent standard error. If *vertical bars* are not visible, the range is smaller than the size of the *symbol*

D-Ala-D-Ala dipeptides [55]. Our results in the axenic cultures are consistent with this situation because the concentration of dissolved combined D-Ala increased in the late exponential phase (see Fig. 1), and its origin could be the rearrangement of the peptidoglycan in the transition to the stationary phase. Recent findings show that D-amino acids have previously unappreciated regulatory roles in prokaryotes [8]. *Bacillus* species regulate the development of spores into vegetative cells by altering the relative concentrations of available D- and L-Ala [33]. Recently, while studying a mutant form of *Vibrio cholerae*, Lam et al. [28] reported that bacteria produce diverse D-amino acids which accumulate at millimolar concentrations in supernatants of stationary-phase cultures. These D-amino acids seem to modulate the synthesis of peptidoglycan by regulating enzymes that synthesise and modify it. These authors consider that this can be a common strategy for bacteria to adapt to changing environmental conditions and, mainly, when resources become scarce. More recently, Cava et al. [8] found that concentrations of 2.0 mM D-Ala stimulated the conversion of rod-shaped cells to spheres, and Kolodkin-Gal et al. [25] found that some D-amino acids cause biofilm dispersal in ageing bacterial communities. Accordingly, in the *Vibrio* cultures analysed in the present study, the accumulated D-Ala could act as signalling molecule that controls processes occurring at high cell densities, probably when nutrients become limited or, in general, when conditions are not appropriate for growth.

The concentration of dissolved combined D-Ala remained high in the stationary phase and until the end of the experiments. Unfortunately, the incubation time was only 70 h; it would have been very useful to extend the incubation in order to determine whether D-Ala was consumed. The peptide permeases show a strong preference for protein-derived peptides containing L-amino acids and α-peptide bonds and

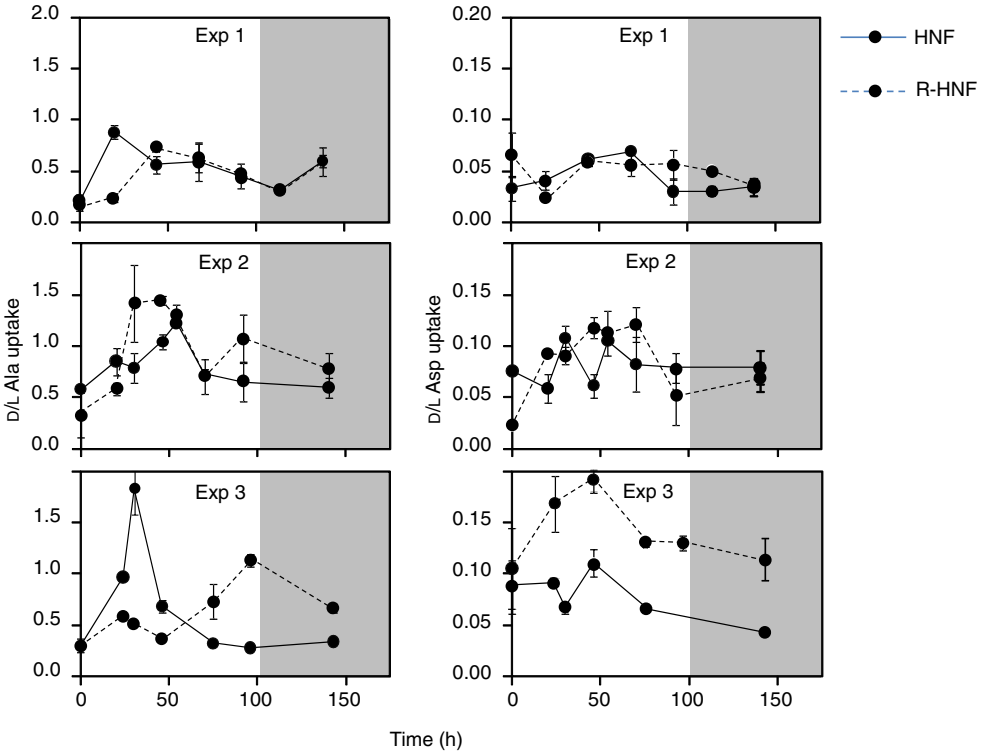

Fig. 5 Time course of the D/L ratio of the uptake of Ala (*left panels*) and Asp (*right panels*) in HNF and R-HNF microcosms in three experiments (*Exp 1*, June 8; *Exp 2*, October 26; and *Exp 3*, November 26). *Grey-shaded area* indicates the time when the R-HNF microcosms showed a high number of HNFs. *Bars* represent standard error. If *vertical bars* are not visible, the range is smaller than the size of the *symbol*

peptides containing D-amino acids, or modified peptide bonds are taken up less efficiently [14]. In our study with natural marine microbial assemblages, prokaryotes were able to easily consume free D-amino acids, but not necessarily the D-amino acids in the combined form as they accumulated in the environment. It is also possible that the release rate of the combined amino acids was higher than the uptake rate, which would result in the accumulation of combined forms. Whether peptides containing D-amino acids are difficult to degrade [8] or are chemically modified, with this modification making their consumption difficult, remains to be determined. In a similar experiment, Gruber et al. [15] found that a pure culture of *Pseudomonas chlororaphis* growing on glucose rapidly produced a complex pool of DOM, and 49 to 68 % of the produced compounds were used within 1 day. The remaining DOM formed a small refractory pool (3 % of the initial carbon). Analysis of the high molecular weight compounds that were released showed that they had predominantly carboxyl and amino groups and that they had originated from amino acids is not unreasonable. Further studies are necessary to understand the differential rates for the removal of combined D-amino acids by bacterioplankton and their interactions with other molecules in the marine environment as well as the way this interaction affects bioreactivity. Recently, Jiao et al. [18] proposed the microbial carbon pump as a conceptual framework for understanding the role of microorganisms in the generation of recalcitrant organic matter and its global consequences for the biogeochemical state of the ocean and climate change. In this context, the chemical characterization of the organic compounds released by prokaryotes becomes particularly relevant.

Other cellular structures, such as capsules, could also be involved in the release of D-Ala to the environment. Stoderegger and Herndl [50] demonstrated that marine prokaryotes are constantly releasing a capsular material into ambient water. In addition, capsules of some Gram-positive bacteria (*Bacillus* sp.) are rich in D-amino acids [12, 33], though, to our knowledge, D-Ala is not a component of the capsular material of Gram-negative bacteria, which are found predominantly in the surface seawater. D-Ala appears to be distributed in several bacterial macromolecules such as teichoic acids, lipopolysaccharides, lipopeptides and siderophores, and some of these compounds are also released during normal growth [20, 31].

As a higher level of complexity, we analysed natural marine microbial assemblages and observed that not only D-Ala but also D-Asp accumulated during the incubation. In these experiments, the number of prokaryotes and concentration of total dissolved D-Ala and D-Asp positively correlated and increased simultaneously in both types of microcosms, HNF and R-HNF. In the R-HNF microcosms, the presence of HNFs could not be avoided completely, and they appeared at the end of the incubations, but a similar correlation was found between the dynamics of the concentration of D-Ala and D-Asp and prokaryote abundance. Nevertheless, HNFs seem to contribute to an increased pool of D-amino acids because the

concentration of D-Ala was slightly higher in the presence of HNFs, and the concentration of D-Ala correlated positively with HNF abundance. The multiple regression analysis also showed that the abundance of HNF influenced the concentration of D-Ala, but to a much lesser extent, that did the abundance of prokaryotes. Protozoa are recognised to exert a significant influence on carbon dynamics, and as a result, their grazing is considered to have an impact on DOM composition. Bacterial cellular components are released to the ambient water as DOM during protist grazing, and several studies have shown that protozoa can release both labile and refractory compounds [3, 7, 38, 51, 54]. Kujawinski et al. [27] identified 80 newly dissolved organic compounds produced by grazing. Our results showed that HNFs are not the main factor influencing the concentration of D-amino acids, which is in accordance with Gruber et al. [15], who found that grazers have a minor influence on the DOM in aquatic systems. Perez et al. [43] examined the concentration of D-Asp during laboratory experiments with surface-water bacterioplankton assemblages in artificial seawater amended with nutrients and found that dissolved total D-Asp concentrations increased during the course of the experiments regardless of whether flagellates were present or not. It is possible that the significance of grazing in the production of D-amino acids varies according to the experimental conditions. Viral lysis could also be responsible for the increased concentration of D-amino acids, as reported by Middelboe and Jorgensen [34], but in the present study, the concentrations of the studied D-amino acids did not exhibit a significant correlation with the number of VLPs.

Another consideration that attributes the production of D-amino acids in the natural microbial assemblages to the growth of prokaryotes is that only D-Asp and D-Ala, in particular, were accumulated in all experiments. Kawasaki and Benner [22] also found relatively high concentrations of D-Ala in DOM released during exponential growth in experiments with coastal bacterial assemblages, whereas other D-amino acid components of peptidoglycan, D-Asp, D-Glu and D-Ser, were not detected or were in low concentration. These authors found differences depending on the aquatic ecosystem. The percentage of D-Glu during growth was elevated only in the experiments with bacterial assemblages collected from the estuary. If the D-amino acids were derived from the breakage or lysis of prokaryotic cells by bacterivorous grazing or viral attack, a mixture of D-amino acids would be released into the environment. Another interpretation of the selective accumulation of these two amino acids is that several amino acids were released but rapidly consumed by the prokaryotic community, and consequently, their concentrations were below the level of detection, whereas some D-amino acids which more refractory to prokaryotic degradation remained in the environment. However, the present study demonstrates that prokaryotes are able to take up free D-Ala efficiently (see Fig. 4), which to the best of our knowledge, is the first report of direct estimations of D-Ala uptake rates by bacterioplankton. The measured uptake rates of free D-Ala were comparable to those of L-Ala because the D/L ratio for the uptake of Ala increased during prokaryotic growth up to values higher than 1 when the availability of D-Ala was high. Some studies [43, 53] have found that prokaryotes shift from preferential L-amino acid uptake to a more efficient utilisation of D-amino acids in the absence of other utilisable organic matter. However, in the case of Asp, the D/L ratio for uptake was always lower than 0.2, which indicates that the analysed prokaryotic community preferentially consumes the L form of this amino acid, even when nutrients become limited, or when conditions are not appropriate for growth.

In the present study, the total D/L-Ala ratio increased, while prokaryotes were growing, in both natural microbial communities and axenic cultures. At the beginning of the experiments in fresh seawater, the values of this ratio were low (<1), but they increased during the incubation and remained high (>3) throughout the experiment (150 h in the natural assemblages). The high dissolved total D/L-Ala ratio at the end of the incubation in the aged seawater indicated that this ratio can be used as an indicator of the biodegradability of the DOM. High ratios would reflect low biodegradability of the organic matter. Other studies have shown that D-amino acids can serve as useful indicators of early stages of diagenesis [21, 24]. Kitayama et al. [24] studied the bioreactivity of the peptidoglycans in seawater and found that the D/L ratio of Ala released from the bacterial cells increased as the diagenetic stage progressed. These authors also suggested that the increase in the concentration of D-Ala was partly due to the release of D-Ala as peptides accompanying the synthesis of new peptidoglycan in the exponential growth phase.

What is the significance of the release of D-amino acids in the carbon flux of the studied system? DOC concentrations were not measured in this study, but assuming the annual mean value of DOC in this surface waters (167 μM) (Azúa et al. in preparation), D-Ala accounted for 0.09 to 0.52 % of DOC which is a high percentage when considering that Benner [4] reported that in marine surface waters, the total amino acids may account for 1–3 % of the DOC. The high concentration of free and combined released D-Ala as a consequence of prokaryotic growth indicates that prokaryotes play an important role as a source of labile and semi-labile DOM in the ocean. In order to know the importance of the extracellular release of D-Ala and D-Asp compared to the production of new biomass, we estimated the carbon production of prokaryotic biomass in R-HNF cultures from the increase in prokaryotic abundance in the cultures by using a conversion factor of 20 fgC cell^{-1} [30]. The released D-Ala and D-Asp represented 3.5–15 and 1.0–12.8 % of carbon produced by prokaryotes, respectively, which indicates the significance of the released C compared to the C produced

as biomass. These percentages are very high, considering that they only correspond to two amino acids, D-Ala and D-Asp, and the prokaryotes likely release some other organic compounds in significant amounts. These results are consistent with those of Kawasaki and Benner [22] who found that the extracellular release of DOM from growing bacteria ranged from 14 to 31 % of the bacterial production. In this context, heterotrophic prokaryotic production rates and growth efficiency would be significantly underestimated if the extracellular release of organic carbon is not taken into account, which would have important implications for the carbon flux in the ocean.

Prokaryotes can produce DOM via two fundamental mechanisms: release during cell death (protist grazing and viral lysis) and release from 'healthy' cells. The relative importance of these two pathways probably varies depending on the environment. In the present study, we demonstrated that the production of dissolved free and combined D-amino acids is significant and attributable under the assayed experimental conditions, predominantly, to release from healthy cells during growth, though bacterivorous grazers can participate in a significant manner. In other experimental conditions, dead cells originating by grazing or even viral lysis can be more relevant as a source of D-amino acids. *V. furnissii* and *V. alginolyticus* release free and combined D-Ala when grown in axenic culture, probably due to arrangements in the peptidoglycan. Free D-Ala is produced by these bacterial species in the initial phase of growth and subsequently consumed, whereas combined forms are released at the end of the growth phase and accumulate in the medium. The significance of D-amino acids in the pool of labile and semi-labile DOM in the ocean and their usefulness as a diagenetic indicator of DOM alterations make it necessary to chemically characterise the molecules that contain them as well as their utilisation and processing by marine prokaryotes.

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