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Dissolved organic matter (DOM) cycling in coral reefs: Compositional constraints

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Master thesis in the form of an NWO proposal according to the guidelines of Environmental Biology: Biomarine Sciences

Supervised by:

Prof. Dr. J.J. Middelburg

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The maximum length of a proposal is 11 pages.

1a. Details of proposal

Title: **Dissolved organic matter (DOM) cycling in coral reefs: Compositional constraints**
Area: √ Geo and Biosphere O from Molecule to Organism
Summary (scientific summary in English, max. 250 words):

The sea hosts one of the largest carbon pools in the world and most carbon is present as DOC. The DOC pool is highly dynamic due to biological activity and large parts of labile DOC are introduced into the food chain via the microbial loop. Recent studies have found that important reef organisms, corals and sponges, also take up and assimilate DOC as a large part of their diet. This underlines the importance of DOC in the energy budget of coral reefs.
It was shown that most DOC produced on coral reefs is highly labile and can be taken up within hours to several minutes. This high turnover complicates the characterization of the labile DOC. Adding to that is the complexity of the coral and sponge holobiont. Even if specific parts of the DOM pool could be characterized, it would be hard to distinguish exact sources or sinks of the organic matter from different parts of the holobiont. Most analytical techniques used for DOM are relatively new and unfortunately information on DOM fluxes and especially composition is still sorely lacking. With this study we plan to investigate (1) the chemical composition of the labile part of DOC used as an energy carrier on coral reefs (2) which compounds are released by different types of reef organisms (3) if these compounds are representative of that type of organism or species specific (4) which other organisms may assimilate this DOC.

1b. Details of applicant

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1d. Renewed application? O Yes √ No
In case of a renewed application please indicate the file number of the previous application and summarize the main changes

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1e. Applying for: √ PhD student O Post Doc O Ship time

1f. Composition of the research group

List all staff members involved in the proposed research: provide name, initials, titles and type of involvement, e.g. daily guidance, technician, thesis supervisor, advisor.

Name and title	Specialization	Institution	Involvement	Hrs/wk
Prof. Dr. Jack Middelburg	Biogeochemistry	Utrecht University	Promotor, daily guidance, thesis supervisor	3
Prof. Dr. Henk Brinkhuis	Marine Paleobiology	Utrecht University / NIOZ	Promotor, advisor	1
Dr. Fleur C. van Duyl	Coral reef ecology	NIOZ	Advisor	2

2. Summary for the general public

(please provide in 100 words a title and summary for the general public, preferably in Dutch)

Opgelost organisch materiaal als energiedrager in koraalriffen
Verschillende organismen op koraalriffen, zoals koralen, sponzen en algen, geven opgelost organisch materiaal af, of nemen dit op als voedingsbron. Uit recent onderzoek blijkt dat sponzen voor een groot gedeelte afhankelijk zijn van opgeloste stoffen voor hun dieet. De precieze samenstelling van dit opgelost organisch materiaal is echter nog grotendeels onbekend.
We zullen daarom de samenstelling van het organisch materiaal onderzoeken, zoals afgegeven of opgenomen door verschillende soorten organismen op het koraalrif. Door het gebruik van soortspecifieke stoffen en moleculaire labels, willen we achterhalen van welke soorten het materiaal afkomstig is en welke soorten het gebruiken als voedingsmiddel.

3. Top 5 publications of the applicant and research group related to the proposed research

1. **Duyl FC**, Moodley L, Nieuwland G, IJzerloo LV, Soest RWMV, Houtekamer M, Meester EH and **Middelburg JJ** (2011) Coral cavity sponges depend on reef-derived food resources: stable isotope and fatty acid constraints. Marine Biology 158, 1653-1666.

2. Wijffels RH, Koopmans M, Rijswijk P, Martens D, Egorova-Zachernyuk TA and **Middelburg JJ** (2011) Carbon conversion and metabolic rate in two marine sponges. Marine Biology 158 9-20.

3. **Duyl FC**, Scheffers SR, Nieuwland G and Bak RPM (2004) Removal of bacteria and nutrient dynamics within the coral reef framework of Curaçao (Netherlands Antilles). Coral Reefs 23, 413-422.

4. Boschker HTS and **Middelburg JJ** (2002) Stable isotopes and biomarkers in microbial ecology. FEMS microbiology ecology 40, 85-95.

5. Rochelle-Newall EJ, Pizay MD, **Middelburg JJ**, Boschker HTS and Gattuso JP (2004) Degradation of riverine dissolved organic matter by seawater bacteria. Aquatic Microbial Ecology 27, 9-22.

4. Description of the proposed research

Max. 4 pages (and max. 3600 words), including figures, excluding literature references). Include details of objectives, innovative aspects, scientific approach, impact, and literature references

Rationale

The sea hosts one of the largest pools of carbon in the world and dissolved organic carbon (DOC) holds almost as much carbon as CO_2 in the atmosphere (Hedges 1992). Dissolved organic matter (DOM) is defined as any organic compound that can pass a 0.2 µm filter. DOM is the most abundant form of organic matter in the oceans (Martin & Fitzwater 1992) and is divided into functional groups; labile, semi-labile and refractory DOM. Refractory DOM is the most common form found in the worlds oceans, due to it's low bioavailability. It consists mostly of the smallest DOM fraction and is not available for bacterial degradation, giving it a residence time of years to centuries (Amon & Benner 1996). The semi-labile part of the DOM is readily used, but less optimal for bacterial growth (Connolly & Coffin 1995) and might require extracellular hydrolysis before it may be degraded by microbes (Anderson & Williams 1998). Semi-labile DOM has a turnover rate of months to years. Labile DOM includes amino acids, sugars and other compounds, which are readily consumed by other organisms and can have a turnover rate of only several minutes (Carlson & Ducklow 1994). Therefore most labile DOM is found in the shallower parts of the ocean, the euphotic zone, where primary production is high (as reviewed by Ogawa & Tanoue 2003).

The three main sources of DOM are phytoplankton exudation (Cole et al. 1982), sloppy feeding and virus-mediated lysis of bacteria and phytoplankton. The most common sink of DOM in the surface oceans is bacterial uptake and transfer to higher trophic levels through the microbial loop (Azam et al. 1983). Bacteria readily take up the labile part of the DOM, transferring it to particulate organic matter (POM) and large parts of organic material that was taken up by bacteria or stored in phytoplankton is released again by means of viral lysis (Brussaard et al. 1995, Wilhelm & Suttle 1999). By ways of this cell lysis, viruses mediate the transition of POM back to DOM. It was found that 3 to 26% of all organic carbon primary production in the oceans is released as DOM due to viral lysis (Wilhelm & Suttle 1999). DOC can also be released upon transfer to higher trophic levels by sloppy feeding, for example by protists feeding on bacterio- and phytoplankton (Christaki et al. 1998, Gonzàlez 1996).

Bacteria are especially dominant in oligotrophic waters (Caron et al. 1995), such as are found around coral reefs. Coral reefs are areas of high gross productivity and biodiversity in oligotrophic waters (Kohn & Helfrich 1957). This 'coral reef paradox' was described by Darwin as early as 1842. It was found that primary production on coral reefs is more dependent on benthic organisms than on phytoplankton in the water column (Grigg et al. 1984). The main driver of the high productivity in low nutrient waters is the symbiosis between invertebrates, such as sponges and corals, and dinoflagellate microalgae. The dinoflagellates, or zooxanthellae, are endosymbionts that live within the cells of hermatypic corals (Hoegh-Guldberg 1999) and many types of sponges (Vicente 1990). Zooxanthellae are primary producers and pass up to 95 % of their production to the host in the form of sugars, carbohydrates, amino acids and small peptides. In turn, the corals provide the algal symbiont with protection and the zooxanthellae can use ammonia and phosphate from their hosts' waste products (Hoegh-Guldberg 1999). Like bacteria and phytoplankton, the zooxanthellae are subject to viral lysis and viral infection might spread rapidly among individuals (Wilson et al. 2001). Corals and especially sponges filter big amounts of water for feeding, which might increase the chance of viral infection due to the number of contacts with viruses (Hadas et al. 2006). By means of this lysis and sloppy feeding by corallivores (and herbivores in the case of algae), DOM is released again in mostly the same way as for the microbial loop. Additionally, many dominant species on the reef have been found to release large amounts of DOM themselves, such as corals (Wild et al. 2004, Tanaka et al. 2009) and macro-algae (Wild et al. 2009).

Coral reefs are located in shallow areas with a high fraction of labile DOM (Ogawa & Tanoue 2003) and the levels of labile DOM in the water increase due to production on the reef. Due to it's high amino acid and saccharide content, the labile part of the dissolved organic matter could prove to be an important energy carrier on coral reefs. This might help to explain the previously observed coral reef paradox of flourishing reefs in seemingly clear oligotrophic waters. DOC has been argued to be a food source for marine invertebrates since the late nineteenth century (Jørgensen 1976). In 2006 Hadas et al. found that sponges can take up viruses, which can easily pass a 0.2 µm filter and are therefore considered to be dissolved (Hadas et al. 2006). Yahel et al. provided proof that a big part of the sponge diet may be comprised of DOC (up to 90%) and showed that DOC was the biggest

source of carbon taken up by the sponge *Theonella Swinhoei* (Yahel et al. 2003). Bacteria associated with sponges have been assumed to take up DOM (Ribes et al. 1999), but direct uptake of amino acids by sponge cells was also found (de Goeij et al. 2008a). Recent studies by the Goeij et al. (2008b) have shown that the removal of DOC by cavity sponges, is two orders of magnitude higher than the removal of bacterioplankton by the same sponges. This indicates that DOC is an important part of the sponge diet. Clearance rates ranked among the highest ever recorded and the amount of labile DOC in an incubation chamber could be filtered within several minutes. It was also shown that the carbon from this DOC is mainly assimilated and respired by the sponge cells (de Goeij et al. 2008a). De Goeij et al. (2008) also found that the sponge assimilated over half of this DOC, though sponge growth was minimal. They argue that most of this DOC must have been exported in particulate form due to a high turnover op sponge cells. This rapid turnover by the sponges chaonocytes was shown in an article from 2009 (De Goeij et al. 2009). This means that sponges, as bacteria, are a link between dissolved and particulate organic matter and that this 'sponge loop' may also feed higher trophic levels. These findings also suggest that different species of sponges might rely on DOC as a major part of their diet. Therefore these sponges may play an important role in retaining energy in the coral reef ecosystem, using DOC as a food source. Tanaka *et al.* (2011) measured DOC concentrations over a daily cycle and found a increase of 1.7 to 3.9 mmol DOC m^{-2} h^{-1} during daytime and a net decrease of 0.8 to 1.9 mmol DOC m^{-2} h^{-1} during night, which indicates a standard cycle of daily production and nightly consumption of DOC on a reef. The DOC concentration over the reef (66 – 75 µmol l^{-1}) was found to be higher than that of the adjacent oceanic surface water (57 – 58 µmol l^{-1}), indicating that coral reefs may serve as a source of DOC, but only a fraction of the DOC was found to be exported from the reef area (Tanaka et al. 2011). This suggests a high retention of energy on the coral reef.

Corals are associated with many different organisms other than zooxanthellae alone. Archaea, bacteria, algae, fungi and protists, have been found living on the coral skeleton, tissue and mucus. The coral animal and all of its parts, combined with all organisms living in association with it in a mutualistic symbiosis, are called the holobiont. The coral holobiont was also found to release DOC (Crossland 1987, Ferrier-Pages et al. 1998) and even before the release of DOC by corals was studied, it was found that the holobiont releases high amounts of mucus which can be used as a food source by other species (Benson & Muscatine 1974, Ducklow & Mitchell 1979). High amounts of mucus are produced as a protection against UV (Drollet et al. 1993), pathogens (Kvennefors et al. 2012) and temporal desiccation on tidal flats (Krupp 1984). This high-energy coral mucus is photosynthesized by the zooxanthellae (Brown & Bythell 2005) and consists of transparent exopolymer particles (TEP) which are mainly large polysaccharides. These TEP aggregate easily due to their sticky nature, but polysaccharides may also be present in seawater in separate molecules as a precursor of TEP (Passow 2002). A study by Wild *et* al. (2004) showed that 56 to 80 % of the mucus excreted by corals enters the DOC pool. Green macro-algae also release TEP. In pilot studies by our research group it was shown that *Dictyota pinnatifida* can enrich seawater with TEP in a 1.5 liter incubation chamber sevenfold within 3 hours (unpublished data by the authors). Findings by Wild *et al.* (2009) confirm a substantially higher release of DOC by macroalgae compared to corals, though results were highly species specific. Keeping the equilibrium between TEP and its precursors in mind, it stands to reason that these algae also release high quantities of DOC. A study by Haas *et al.* (2010) shows that other common types of algae as *Halimeda opuntia* and *Caulerpa serrulata* release organic matter as well. It was found that these algal species release 2.29 mg of organic carbon per hour in daylight conditions and that over 90 % of this organic carbon is released as DOC. From the total organic carbon 59 % was found to consist of carbohydrates and 32 % consisted of proteins (Haas et al. 2010), which indicates that the organic matter released by macro-algae is highly labile. We performed trials with macro-algae enriched seawater and we found that excavating sponges may take up substantial amounts of TEP and DOC from the surrounding water within minutes (unpublished data by the authors).

Corals were not only found to release DOC, but a net uptake of DOC was also measured under low light conditions (Naumann et al. 2010) and even during the day. Haas *et al.* (2010) investigated DOC release by three different types of Caribbean sclerentinian corals and found a net release rate by two *Porites* species, while *Manicina sp.* showed a substantial net uptake of DOC. This DOC uptake was also shown in older studies and might be linked to microbes in the holobiont (Ferrier-Pages, Gattuso et al. 1998). Different genera of corals may harbor different bacterial communities (Rohwer et al. 2002), which may result in different rates (Naumann et al. 2010) and in all probability a different of composition of the released DOC. The different types of zooxanthellae

present in the host may also contribute to the species-specific uptake and subsequent release of DOC by corals (Al-Moghrabi et al. 1993).

Objectives

All of the uptake and release of DOM discussed underline the importance of DOC in the energy budget of the coral reef. DOC is produced by many different organisms living on or in association with coral reefs, such as corals, sponges, algae and bacterioplankton. And it is consumed by dominant reef species, namely corals, sponges and free-living bacterioplankton. It was also shown that most of the DOC produced on coral reefs is highly labile and can be taken up by consumers within hours to several minutes. This high turnover of DOC within the coral reef food web, makes it difficult to characterize the labile part of the DOC. The complexity of the coral and sponge holobiont as reviewed by Bourne *et al.* 2009 (Bourne et al. 2009) further complicate investigations into reef derived DOM. Even if we could characterize specific parts of the DOC pool, it would be hard to distinguish the exact source or sink of the organic matter from different parts of the holobiont. Most analytical techniques used for DOC today are relatively new and unfortunately information on DOC fluxes and especially DOM composition is still sorely lacking. With this study we plan to (1) investigate the chemical composition of the labile part of the DOC used as an energy carrier on coral reefs (2) determine which compounds are released by different types of reef organisms (3) determine if these compounds are representative of that type of organism or more species specific (4) investigate which other organisms may assimilate this DOC. We will shortly discuss some of these techniques and previous findings that are relevant for the proposed research before we present our scientific approach.

Techniques

Most recent studies have focused on quantifying DOC stocks and fluxes on the reef and they examine total carbon fluxes, rather than the chemical composition of dissolved organic carbon. Often O_2 consumption is used as a proxy for (microbial) DOC degradation (Haas et al. 2010). For an accurate DOM quantification samples are usually analyzed with a total carbon analyzer and total nitrogen analyzer. From the available literature, it would seem that DOC release and uptake rates by different reef organisms are highly variable (Ferrier-Pages et al. 1998, Yahel et al. 2003, Brown & Bythell 2005, de Goeij et al. 2008b, Tanaka et al. 2009, Wild et al. 2009, Haas et al. 2010, Naumann et al. 2010, Tanaka et al. 2011) and influenced by environmental factors (Bednarz et al. 2012).

When analyzing the chemical composition of DOM in a seawater sample, the first major difficulty to face is to obtain the organic compounds from solution. There are three methods used to isolate DOC from seawater; freeze drying, ultrafiltration and solid-phase extraction. A method successfully used in freshwater biochemistry involves freeze drying and subsequent analysis of the solid phase (Schiff et al. 1997). This method was also used to analyze seawater samples (Fry et al. 1996), but the main issue with this method of obtaining DOM from seawater is the high concentration of salts that are left after the water is removed. Added to that is the comparable low concentration of DOC in these samples. Not only is the amount of DOC in a seawater sample comparatively low, but when a sample is combusted the salts may form complexes with the DOM and aggregate. The salts can bind to the DOM, making it unsuitable for further qualitative analysis, or to the CO_2 that is released when combusting the sample for quantitative analysis (Fry et al. 1996).

A second method to enrich or isolate DOM is the use of ultrafiltration. Ultrafiltration, like any other type of filtration, selects particles based on size. With this method, particles of only 1 kDa may be filtered from the sample over a semi-permeable membrane. Salts from the seawater will be able to pass this membrane, while DOM compounds bigger than 1 kDa will be caught on the membrane. Usually the sample is pre-filtered on coarser filters prior in order not to clog the ultrafiltration filter units (Rochelle-Newall et al. 2004). After the ultrafiltration the DOM retained on the filter may still form aggregates and some salts will still be present in the collected fraction. It is however possible to desalt the DOM fraction by diafiltration with high-purity water. Due to the high amount of possible compounds present in the collected DOM sample, it seems impossible to find a proper solvent to extract these unknown organic compounds, so the collected DOM fraction is usually analyzed as a whole. One of the advantages of ultrafiltration is the separation of organic material into different size fractions, which are an important characteristic of DOM. A practical division was made into two main fractions based on size; a low (LMW < 1 kDa) and a high molecular weight

fraction (HMW > 1 kDa). LMW fractions were found throughout the water column in the ocean, while HMW fractions were found in higher concentration in surface water, which suggests that DOM with a high molecular weight is more labile (Ogawa & Tanoue 2003). Bioavailability is of course not purely based on the size class of the particles, but it was found that bacteria can utilize a significant larger fraction of the HMW DOM than of the LMW fraction (Amon & Benner 1996). A similar trend was shown by Rochelle-Newall *et al.* (2004) and they showed that changes in amino acid composition during an incubation are a good indicator of total organic matter degradation by bacteria. Suzuki *et al.* (2000) found that that the HMW fraction of the DOC was more abundant in a coral reef lagoon, than in the surrounding waters. Tanaka *et al.* (2011) found from bacterial decomposition that 77 % of reef derived DOC was easily degraded, or labile. This again leads to the assumption that reef derived DOC is highly labile and mainly consists of HMW fractions. Therefore ultrafiltration seems especially suited for this study, as the HMW fraction is likely to be a more probable candidate for energy transfer in coral reef ecosystems.

The third method for obtaining DOM from seawater is solid-phase extraction. The method is based on hydrophobic interactions between the DOM and a nonionic porous sorbent. Many different sorbents are available, of which XAD resin is the most commonly used (Aiken 1985). Due to the method of adsorption, only a fraction of the DOM can be obtained by solid-phase extraction with a strong bias towards the hydrophobic part of the DOM. Moreover, the DOM needs to be prepared for adsorption by lowering the pH and is released again from the resin by adding NaOH. In this process the composition of the DOM might be affected and compounds other than the DOM might be released from the resin, contaminating the sample. Nevertheless, these resins have high adsorption capacities, so the advantage is that large volumes of water may be processed and the use of a combination of resins might increase the fraction of DOM that can be isolated from the water (Lepane 1999). The advantage over ultrafiltration is that the LMW fractions can be extracted as well. Another advantage compared to other extraction methods is that the obtained DOM is pure and free of salts.

Once the DOM is collected it can be further analyzed. Isotopic composition for example can be measured by ways of mass spectrometry. Isotopic composition is a well accepted tool, used for the reconstruction food webs and had also been used to retrace the origin of DOC consumed by reef cavity sponges (van Duyl et al. 2011). Another measurement is the C:N ratio of the DOM, which is an important factor for biological processes in terms of possible nutrient limitation. The C:N ratio of the HMW fraction can be measured directly with a CHN analyzer after ultrafiltration. The C:N ratio of total sample may also be measured with a CHN analyzer and can be compared to that of HMW fraction. Alternatively, the C:N ratio of a total DOM sample can be calculated with the HTC method or as discussed earlier by burning the sample in a total organic carbon analyzer. Suzuki *et al.* (2000) compared DOC and DON values after ultrafiltration and found that the HMW DOM produced on coral reefs has a high nitrogen content and a remarkably low C:N ratio of approximately 6 to 8. This in contrast to oceanic surface waters, where nitrogen is more limiting.

Other frequently used methods to determine molecular composition are gas or liquid chromatography mass spectrometry (GC-MS or LC –MS). Before the isolated DOM is analyzed by chromatography it is usually hydrolyzed or oxidized into monomers that can be identified with this technique. This does create a bias towards compounds that are readily hydrolyzed into monomers and certain compounds as ribose may be destroyed during hydrolysis. The chromatograph separates the different molecules based on their melting point or vaporization and then passes them through a column coated with polymers that interact with the molecules. Because of the different interactions each molecule has with these polymers, each molecule will take a different time to pass through the column. These differences in retention time in the chromatograph do not only tell us something about the size and charge of the molecules, but it also means that different compounds are passed to the mass spectrometer separately. Here the molecules are ionized into smaller fragments and caught on detectors that can determine molecular mass and quantity. By analyzing the pattern of these fragments, it is possible to detect known compounds whose spectra are stored in a database. Profiles exist mostly for amino acids and carbohydrates, which are high energy compounds, that may be important in energy cycling on the coral reef. A structural formula can also be distilled from the weights of the entire molecule and the ionized fragments if a spectrum is not found in the database, in order to characterize previously unidentified substances. Again the biggest issue with extraction of DOM from seawater is the difficulty to obtain separate DOM molecules. Therefore we can only examine hydrolyzed parts of the total DOM sample using

GC-MS or LC-MS.
Some organic substances might have the same retention time in the chromatograph causing them to elute at the same time, which makes it difficult to analyze these substances with the help of mass spectrometry. As marine DOM is still largely uncharacterized, most spectra for DOM are not yet stored in databases, therefore only the common parts of the DOM can be easily identified. Haas *et al.* (2010) for example studied the carbohydrate and lipid content of algal derived dissolved organic matter and they found that approximately 59 % of the organic matter consisted of carbohydrates, 77 % of which was glucose. 32 % Of the organic matter they analyzed consisted of proteins, which means that 91 % of the organic matter consisted of highly labile DOM. So while GC-MS does not uncover the true composition of separate molecules of DOM, it can provide a clear characterization of the DOM sample and it's energetic potential.

A second technique available to analyze the molecular structure of DOM is nuclear magnetic resonance (NMR). A technique in which the atomic nuclei of a substance are targeted with electro-magnetic radiation which is re-emitted by these nuclei at a specific resonance, depending on the electron distribution or bonds with neighboring atoms. From the resonance spectrum, specific functional groups (f.e. acid groups, ketones, aromatic rings etc.) may be recognized and quantified. Though this method does not yield detailed composition of the DOM (only functional groups) it has the advantage of analyzing the entire DOM pool and no hydrolysis is required. Therefore NMR is ideally used to compare chemical composition between different DOM fractions and may be used for example to quantify the presence of highly energetic functional groups within a sample. The purification of the DOM sample is still an issue using this technique, so again only functional groups of the collective DOM sample, mainly the HMW fraction after ultrafiltration, can be analyzed. In 1997 Aluwihare & Repeta found from ^{1}H and ^{13}C NMR that HMW DOM consists mainly of carbohydrates and especially acyl oligosaccharides (Aluwihare & Repeta 1997). From ^{15}N NMR it was shown that 70 to 90 % of the HMW fraction consists of amides and ^{31}P NMR showed that phosphorous was mainly present in the form of esters or phosphonates (McCarthy & Pratum 1997, Kolowith et al. 2001). These substances were found in most HMW DOM samples analyzed and showed extremely little differences over spatial or temporal scale as reviewed by Ogawa & Tanoue (Ogawa & Tanoue 2003). This again shows the high energy potential of HMW DOM.

A novel way to analyze protein composition in low quantities and with high accuracy is with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) (Witt et al. 2003, Kujawinski et al. 2002) . During this type of mass spectrometry analysis, an oscillating electrical field is in place perpendicular to the magnetic field. By a combination of these two forces working on the ions they go into a higher state of kinetic energy and start moving in phase in a predictable pattern (cyclotron). The frequency of the movement can be calculated to a mass spectrum, with a so-called Fourier transformation. The resulting values are a ratio between the charge of the ions and their mass. The major downside to the use of this technique is the fact that samples need to fully purified and free of salts in order to be analyzed. Therefore solid-phase extraction is used to obtain purified DOM from seawater (Koch et al. 2005). Several thousand molecular formulas of LMW compounds were found by Koch *et al*. (2005) and they were used to compare DOM from a terrestrial origin and DOM from marine waters. The authors found striking resemblances in compound composition and bond structure between DOM collected from the two very distinct sources and concluded that marine DOM probably has a higher terrestrial origin than previously thought and that refractory DOM has certain common structural features that make it inaccessible or unattractive for microbial degradation. The chance of determining the structural formula for a certain substance might be further enhanced by first separating the DOM from the solid-phase extraction with the help of chromatography (Koch et al. 2008).

Labeling of organic matter is a technique used to pinpoint either the source or sink of organic matter and can be used to show which part of an organism, or which part of the holobiont, has stored or incorporated this organic matter. Wijffels *et al.* (2011) for example, fed ^{13}C labeled diatoms to two species of temperate water sponges and showed that the labeled diatoms were rapidly taken up by the sponges and that the labeled material was slowly incorporated in sponge-specific fatty acids. This provides proof that the diatoms are taken up by the sponge and that the sponge can utilize these diatoms as a food source. This seems straightforward, but by labeling DOC it can be proven which part of the holobiont is responsible for the uptake of dissolved matter and if it is incorporated in the sponge or coral tissue or only in bacteria or archae. Ferrier-Pagès *et al.* (1998) for example, added ^{14}C labeled bicarbonate to an incubation chamber with the

sclerenrtinian coral *Galaxea fascicularis*, which then produced ^{14}C labeled DOC. Subsequently uptake of ^{14}C labeled organic material by free-living, epibiotic and intracellular bacteria was investigated with and without the addition of antibiotics. Only the treatment without added antibiotics yielded an uptake of labeled bicarbonate by the coral holobiont, suggesting that bacteria serve an important function in the uptake of DOC (Ferrier-Pages et al. 1998). The use of species-specific compounds is another way to distinguish the source of dissolved organic material. Known compounds produced by specific species or family can be identified within a sample to determine the origin of the DOM, as was done for example by van Duyl *et al.* (2011) who studied the food source of cavity sponges. By using fatty acid biomarkers, they found that the main source of food for these sponges was derived from DOM produced by corals and crustose coralline algae on the reef and not by phyto- or bacterioplankton from the open sea.

Scientific approach

With the proposed study we plan to examine the chemical composition of DOC used as an energy carrier on coral reefs and we want to pinpoint the sources and consumers of this organic material. We plan to perform all fieldwork on Curaçao in the Caribbean. First and foremost because a great deal of research on coral reefs (and DOC) has already been done on the island and we can use prior findings and compare our results with other studies. Secondly, we plan to cooperate with several other scientists on site and finally proper research facilities are present on Curaçao with easy access to the coral reef.

In order to asses the producers and consumers of DOC on coral reefs we plan to study the uptake and release of different species of corals, sponges and algae in incubation experiments, as they are the dominant species of Caribbean and most other coral reefs. We also plan to use different types of cyanobacteria, for they form extensive mats on the reefs around Curaçao, they are autotrophs and their ability to fix nitrogen might result in a production of DOM that is distinctly different from that produced by other reef organisms. Research was done into the release of compounds by cyanobacterial mats (Stal 1995), cyanobacteria in stromatolites (Goh et al. 2010) and planktonic cyanobacteria in reef environments (Ferrier-Pages & Gattuso 1998) and Salihoglu *et al*. (2008) found that planktonic cyanobacteria in the Sargasso Sea were responsible for 33 % of the carbon production in the area. To our knowledge though, not much data is available on DOM release by cyanobacterial mats on coral reefs to date. We plan to use at least two different species of corals, sponges, algae and cyanobacteria, so we may examine the DOM composition of the different species for differences and similarities within each group. For the coral species we plan to use *Madracis mirabilis* and *Montastrea annularis* as they were used successfully during DOC release trials by the research group, they are common species on Caribbean reefs and easy to collect without damaging the reef. Two algal species that were found to produce high amounts of organic matter are *Dictyota pinnatifida* (unpublished data by the authors) and turf algae (Wild et al. 2009) , are suited candidates for the same reasons. *Halisarca caerulea* will be used as one of the sponge species, as it is a common cavity sponge in the Caribbean that is known to take up high amounts of DOC in a short period of time. A second sponge species will be selected during trials with net DOC uptake. Similar trials will be held with net production of DOC by cyanobacteria, as not much literature is available on DOC production by cyanobacterial mats in coral reefs. All species will be collected from the reef a month prior to the experiments and placed in flow-through aquaria to recover and their health will be monitored.

Incubations will be performed in 0.2 µm pre-filtered seawater in order to minimize the effect of free-living microbes on the DOM concentration and composition in the incubation chambers and to ensure that only DOM can be taken up by the incubated organism. TEP will be quantified in ambient seawater. It was found by Passow (2002) that TEP is at equilibrium in seawater and that it is replenished after it is filtered out with a 0.2 µm filter. If the aggregates can be caught on a 0.2 µm filter, while the precursors of TEP can be filtered through, this means that by definition TEP can be considered to be both particulate and dissolved. We believe that the amount of TEP present in seawater in mucoid form should always be taken into account when determining DOC concentrations in order not to underestimate the total amount of bioavailable DOC. Experiments will last for a maximum of 15 minutes as uptake and release of DOC was found to be significant within these time frames and to minimize potential changes in DOC composition due to microbial degradation. The amount of DOC released or taken up is always a net flux, so some DOC might have been produced during the experiment, which was taken up again by other organisms before the end of the experiment. Labile parts of the DOC can be transformed by microbial activity, as bacteria can utilize large parts of the labile DOC and produce refractory DOC within hours (Ogawa

et al. 2001). To minimize stress to the test organisms, they will be transferred from the flow-through into a glass beaker containing 0.2 µm pre-filtered seawater to be rinsed before they are gently placed into the incubation chambers. A seawater sample will be collected from the 0.2 µm pre-filtered seawater prior to the start of the incubation to compare DOM concentration and composition at the start of the experiment and at the end of the experiment. By doing so, the quantity and quality of the DOM production or uptake can be determined. An external light source will be used to control irradiance throughout the experiment and light levels will be measured throughout the experiment as a control.
In order to quantify the fluxes of the measured compounds the total DOC and DON concentration will be measured in a total carbon analyzer. From these samples the C:N ratio of the DOC can be calculated. We will use 0.2 µm polycarbonate filters to obtain these DOC samples, as this is the size for which particles are defined to be dissolved. Many studies were performed using GF/F (0.7 µm filters) or 0.45 µm filters, but when using coarser filters like GF/F, 35 to 43 percent of the total bacteria count will still be included in the filtrate (Lee et al. 1995). Prior to filtration the polycarbonate filters will be acid washed three times to prevent contamination of the DOC samples with carbon that may be released from the filters. All DOC samples will be acidified as soon as possible (to pH<2 with hydrochloric acid) to stop microbial activity and simultaneously transform all inorganic carbon to CO_2, so only organic carbon will measured in the total carbon analyzer. Additional filtrations of the seawater on 0.2 µm polycarbonate filter will be performed at t_0 and t_{end} and TEP concentration will be determined colorimetrically (Passow & Alldredge 1995) to account for the concentration of TEP (and a calculation of TEP precursors) in the DOC sample. The total concentration of TEP will be calculated as a possible energy carrier that can be taken up in either dissolved or particulate form.

At t_0 and t_{end} of each incubation a water sample will be taken from which the HMW fraction of the DOM will be collected during ultrafiltration with a 1 kDa filter size. This is the most interesting fraction in term of energy budgets on the reef, as the HMW fraction is most easily degraded by microbial activity and arguably the most labile fraction. The collected HMW fraction will be tested for the presence of functional groups and isotope composition by ways of gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). The analysis of functional groups will tell us more about the chemical composition of DOM as an energy carrier on coral reefs, while the isotopic composition of the sponge, coral or algal tissue and endo- and epibionts will be tested, to confirm the source of the DOM and will provide us with the tools to reconstruct a 'DOM food web' for the coral reef. Part of the sample will be analysed with NMR to show differences in functional groups between t_0 and t_{end}. We will perform a solid-phase extraction of the incubation water at t_{end} so that samples may be analysed with FT-ICR MS. The obtained structural formulas of part of the DOM can be discerned for different producers and in all probability will yield us new insights into species specific characteristics of DOM. Finally we plan to subject the DOM samples to microbial degradation to calculate the percentage of produced organic matter that is bioavailable.

Next to the first incubation study, we plan to perform extensive labeling studies, administering ^{13}C labeled carbohydrates or bicarbonates and ^{15}N labeled nitrates to our algae and coral species over longer periods of time in a controlled lab environment. During intensive labeling we will collect tissue samples of the algal and coral tissue at different time intervals and isolate microbes living inside or in association with the coral. We will examine the tissue of all these organisms to see where uptake has taken place and if the labeled substances have been taken up in their tissues. In this way we want to confirm the location of DOM uptake. Once the label is sufficiently expressed in the algal and coral tissue, we will collect labeled DOM produced by these organisms by means of ultrafiltration and analyze part of the labeled HMW fraction collected in the same way as was done for the incubation experiments. The isotopic label will make it easier to discriminate between DOM produced by the algae or corals and background DOM that was already present in the filtered seawater prior to incubation, when analyzing the samples with mass spectrometry.

Another part of the collected labeled HMW fraction we will use in cross-species experiments. To this end will use a second round of incubation experiments, with much the same set-up as the first round of incubation experiment. The exception in this case is that we will only incubate the two sponge species and we will add the labeled HMW DOM collected from our algal and coral species. This way we want to test how much of the produced labile fraction of DOM on the reef is utilized by other reef organisms and we want to show which parts of the sponge are responsible for this uptake. We will take tissue samples of both our sponge species, isolate the microbes living inside

or in association with the sponge and determine which part of the holobiont was responsible for the DOM release and/or uptake.

Innovative aspects

With this study we will advance our limited understanding of net release and uptake of DOM by reef organisms by means of a thorough characterization of the DOM that plays a key factor in coral reef functioning. We will gain new insight into the composition of DOM with NMR techniques and GC-MS and hope to discern different patterns in the molecular composition of DOM released by different species. These species-specific signatures may be used as a tool for future DOM studies on coral reefs.
This is also the first time a study has been proposed into DOC release rates by benthic cyanobacterial mats on coral reefs, combined with a characterization of species-specific compounds. This part of the research could gain new insights into the functional role of cyanobacteria on coral reefs and may provide novel insights into the specific compounds released by these bacteria.
To our knowledge, this is the first time a study focuses on the integration of both quantitative and qualitative DOC fluxes within the coral reef ecosystem, including all main groups of reef organisms important to this flux; corals, algae, sponges and cyanobacteria.

Impact

A proper understanding of the nature of DOM is essential for management of coral reef ecosystems. DOC has proven to be an important part of energy cycling on these reefs and a characterization of the compounds involved in the energy retention on coral reefs is key to a better understanding of coral reef ecosystem functioning. If funded, this would be the first major project to unravel the composition of DOM on coral reefs, supported by collaborations with highly skilled professionals and well-equipped research facilities both nationally and internationally.

We expect to produce at least 3 publications articles in high-ranking journals at least 1 of which in Science or Nature. Since the findings of this research can provide important tools for future research, we expect a high impact and high citation of these articles.

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5. Timetable of the project

Assuming that the project can start in January 2013, we adopt the following time schedule:
Year 1 (2013)
- (Jan – Jul) Reading literature, ordering materials, implementation and optimalization of the methods and preparation for fieldwork
- (Aug – Dec) Fieldwork

Year 2 (2014)
- (Jan – Mar) Isotope and chemical analysis and data processing
- (Apr – May) Writing a scientific paper
- (Jun – Jul) Preparation for second fieldwork period
- (Aug-Dec) Fieldwork

Year 3 (2015)
- (Jan – Mar) Chemical analysis and data processing
- (Apr – May) Writing a scientific paper
- (Jun – Jul) Preparation for third fieldwork period
- (Aug – Oct) Fieldwork
- (Nov – Dec) Chemical analysis and data processing

Year 4 (2016)
- (Jan – Feb) Writing a scientific paper)
- (Mar) Preparation for fourth fieldwork period
- (Apr – Jun) Fieldwork
- (Jul – Aug) Chemical analysis and data processing
- (Sep – Dec) Finalizing manuscript and completion of PhD thesis

The time planning of the fieldwork may change depending on logistic constraints.
The first three fieldwork periods will be planned within the same time period each year, as seasonality is an important driver for DOC release (Naumann et al. 2010).

6. Scientific embedding of the proposed research

Affiliation with national and international research programmes, national and international collaborations

The PhD working on the proposed research, will be supervised by specialists from Utrecht University and the royal NIOZ. Lab facilities of both institutions will be used. The student will be working in close relation with another PhD student of the royal NIOZ on site, who is working on net DOC release and net DOC uptake by coral reef organisms. This nicely complements our biogeochemical work, as net release and uptake rates could later be used to calculate total fluxes. If funded, we will work in close relation with Acroporanet, a consortium of Dutch research institutions working on tropical marine biology, who have an ever-growing database of data collected in coral reef areas in general and have specific knowledge on the reefs surrounding Curaçao. By sharing our findings of energy carriers and energy budgets on reefs with Acroporanet, we hope that they can use this knowledge for a better use and management of coral reef resources. We will collaborate with the international FORCE research group, that aims to combine biological research with social and economical research for a better management of Caribbean coral reefs. All field work will be done in close cooperation with the Carmabi research centre in Curaçao.

All data gathered during this project will be made publically available for third parties through peer reviewed articles and through the PhD thesis.

7. Societal significance

Coral reefs ecosystems are hotspots for marine biodiversity and are one of Earths ecosystems with the highest productivity in areas that would otherwise be marine deserts. Apart from their aesthetic beauty, many people are dependent on coral reef productivity for food, work, protection of their coastline and income from tourism. Almost a third of all fishes are found on coral reefs and it is estimated that about 10 % of global fish consumption is caught in reef areas. At least tens of millions of people are dependent on the production of coral reefs, either professional or as a source of their daily protein consumption. The latter being very important in developing countries. Additionally, several active components important to the pharmaceutical industry have been derived from compounds produced by corals, sponges, molluscs, macro-algae and sea anemones. Other reef organisms, such as macro-algae have been harvested from the reef and are now being cultured as a food source.

Because of climate change due to rising CO_2 levels in the atmosphere, many recent studies focus on carbon fluxes and active pools of carbon. The largest active carbon pool on earth is marine dissolved organic matter. If only 1 % of the marine DOC pool would be oxidized this would be a greater flux of CO_2 than the total burning of fossil fuels. This means there is a pressing need for a better understanding of DOC. Because even though marine DOC is the largest active carbon pool of organic matter on earth, it is still the least understood.

Many studies have been done into marine food webs and particulate organic matter. DOC might prove to be the most important energy carrier in the coral reef ecosystem. Therefore an accurate characterization of DOC on the coral reef is the key to a better understanding of the functioning of the ecosystem as a whole and might provide us with new tools for a better management of these valuable ecosystems.

8. Budget

		Year 1	Year 2	Year 3	Year 4
Personnel (mm)		12	12	12	12
Research costs (k€)					
	Equipment	17			
	Consumables*	8	8	8	8
	Fieldwork/Travel*	5	5	4	4

* The sums requested for consumables and fieldwork/travel expenses combined should not exceed 50,000 euro for the entire grant period.

Specification of the requested funds:
Personnel: 1 Full time PhD student
Equipment: Ultrafiltration equipment
Consumables: Bench fee 5 k, filtration columns for the ultrafiltration device, GF/F and 0.2 µm filters, glass vials, chemicals and gasses and a contribution for the use of the total carbon analyzer, gas chromatography and NMR machinery at the royal NIOZ lab facilities.
Fieldwork/Travel: Rent of the Carmabi lab facilities for 5 months in the first two years, 3 months in year three and four and one return ticket to Curaçao for each of the 4 years. Also included are two return tickets for the coral reef symposium.

9. Financial assistance from other sources

No other financial resources are available to run this research. However, Utrecht University has invested significantly in purchasing and maintaining high-quality analytical facilities such as the Gas Chromatography-Mass Spectrometer, Liquid C-MS, GC-C-IRMS and nanoSIMS. The chemistry

department at Utrecht University posesses NMR equipment that may be used for this research. The royal NIOZ also possesses CG-MS and LC-MS machinery and several Shimadzu TOC and TON analyzers. A tangential Flow Filtration apparatus is available at NIOZ, but a new device will be purchased to prevent contamination.
Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) would have to be performed in agreement with the Max Planck Research Group for Marine Chemistry, Oldenburg, Germany.

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10. Statements by the applicant

YES I endorse and follow the Code Openness Animal Experiments (if applicable)

YES I endorse and follow the Code Biosecurity (if applicable)

YES I have completed this form truthfully

Name: P.J.M. van Leent
Place: Utrecht, the Netherlands
Date: 30-7-2012

Please submit the application to NWO in electronic form (pdf format is required!) using the Iris system, which can be accessed via the NWO website (www.iris.nwo.nl). The application must be submitted from the account of the main applicant. For any technical questions regarding submission, please contact the IRIS helpdesk (iris@nwo.nl).
