Vol. 519: 141–152, 2015
doi: 10.3354/meps11091

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published January 20

# Seasonal variation in dinitrogen fixation and oxygen fluxes associated with two dominant zooxanthellate soft corals from the northern Red Sea

**Vanessa N. Bednarz[1,*], Ulisse Cardini[1], Nanne van Hoytema[1], Mamoon M. D. Al-Rshaidat[2,3], Christian Wild[1,4]**

[1]Coral Reef Ecology Group (CORE), Leibniz Center for Tropical Marine Ecology (ZMT), Fahrenheitstr. 6, 28359 Bremen, Germany
[2]Laboratory for Molecular Microbial Ecology (LaMME), Marine Science Station, Aqaba 77110, Jordan
[3]Department of Marine Biology, The University of Jordan-Aqaba Branch, Aqaba 77110, Jordan
[4]Faculty of Biology and Chemistry (FB 2), University of Bremen, 28334 Bremen, Germany

ABSTRACT: Dinitrogen ($N_2$) fixation by specialized prokaryotes (diazotrophs) represents an important source of bioavailable nitrogen (N) in the ocean. In coral reefs, several substrates and organisms are associated with diazotrophs, but potential $N_2$ fixation activity by zooxanthellate soft corals has not yet been investigated. Such soft corals may contribute importantly to the input of new N into the reef ecosystem, as they can cover substantial benthic areas in today's coral reefs. Therefore, this study investigated $N_2$ fixation of 2 dominant zooxanthellate soft coral groups (*Sarcophyton* sp. and Xeniidae) in a northern Red Sea fringing reef during all 4 seasons of 1 yr. This was supplemented by respirometry incubations and *in situ* monitoring of key environmental parameters. Findings revealed detectable $N_2$ fixation for both soft corals during all seasons. Annual $N_2$ fixation by *Sarcophyton* sp. was 3 times higher than that by Xeniidae, but both soft corals exhibited similar seasonal patterns. $N_2$ fixation significantly increased during summer, when water temperature and light intensity were highest and inorganic nutrient availability was lowest. Coral respiration also peaked during summer and was positively correlated to $N_2$ fixation, while photosynthesis revealed maximum rates during the nutrient-enriched spring season. Given the importance of N for reproduction and growth, $N_2$ fixation may be a key component of soft coral nutrition during summer, when inorganic nutrient availability in the water column is lowest and likely not sufficient to sustain the high metabolic demand of soft corals.

KEY WORDS: Diazotrophy · Xeniidae · *Sarcophyton* sp. · Acetylene reduction · Photosynthesis · Respiration



## INTRODUCTION

Nitrogen (N) is a major limiting nutrient for primary productivity in oligotrophic coral reefs, with dissolved inorganic nitrogen (DIN) concentrations often below 1 µM (Hatcher 1990). Therefore, several reef organisms have evolved physiological mechanisms to conserve, recycle and collect the essential N (Szmant et al. 1990, Tanaka et al. 2006). Many symbiotic cnidarians (e.g. scleractinian corals) show a bi-directional translocation of N compounds between the zooxanthellae and the host. There, the zooxanthellae use the waste N compounds of their host (mostly in the form of ammonium), assimilate it into amino acids and then translocate a portion of them back to the animal (Muscatine & Porter 1977, Rahav et al. 1989). In addition to this inner recycling loop, several benthic organisms (e.g. scleractinian corals, sponges) have evolved a mutualistic symbiosis with dinitrogen ($N_2$)-fixing microbes (diazotrophs) that

*Corresponding author: vanessa.bednarz@zmt-bremen.de

are able to convert atmospheric $N_2$ into bioavailable N (Fiore et al. 2010, Cardini et al. 2014). Up to 60% of N fixed by diazotrophs can be released as dissolved organic N, thereby making it available for organisms unable to fix $N_2$ (Williams & Carpenter 1997). A recent study on scleractinian corals associated with diazotrophs demonstrated that zooxanthellae are the primary users of the $N_2$ fixation products, implying that this process is important for coral photosynthesis and primary production (Lesser et al. 2007). Therefore, the ability of corals to overcome N limitation through $N_2$ fixation may determine their success in oligotrophic waters, ultimately influencing their ecological distribution and abundance on coral reefs (Fiore et al. 2010).

$N_2$ fixation in scleractinian corals has been described in several studies (Williams et al. 1987, Shashar et al. 1994a, Lesser et al. 2007), but there is a paucity of data available for soft corals, despite their common occurrence in tropical coral reef habitats (Benayahu & Loya 1981, Fabricius 1997). At present, $N_2$ fixation rates have been quantified for only 2 octocoral species (*Tubipora musica* and *Parerythropodium f. fulvum*), including 1 azooxanthellate soft coral (*P. f. fulvum*; accepted name: *Rhytisma fulvum*) (Shashar et al. 1994b), while no data are available for zooxanthellate soft corals.

Some soft corals display opportunistic life history features such as fast growth rates, high fecundity and asexual reproduction. These traits can give soft corals an ecological advantage compared to other benthic organisms that allows them to rapidly colonize large areas of a reef. Combined with environmental disturbance, this could result in benthic community shifts from hard coral- to soft coral-dominated reefs, which have been observed at several reef locations worldwide (Tilot et al. 2008, Norström et al. 2009). In the northern Egyptian Red Sea, hard coral cover declined by 5 to 25% between 1996 and 2002, with a concurrent increase in zooxanthellate soft corals of the families Xeniidae, Nephteidae and Alcyoniidae (Tilot et al. 2008). Thus, as soft corals in reefs of the northern Red Sea are becoming an increasingly dominant benthic functional group, they may contribute significantly to $N_2$ fixation, an important biogeochemical process within coral reef functioning. However, little is known about the capacity and contribution of soft corals to $N_2$ fixation within the reef and, furthermore, about the relationship of their $N_2$ fixation with photosynthesis and environmental factors.

Several environmental factors including light, temperature, oxygen ($O_2$) concentrations and nutrient availability can affect marine $N_2$ fixation (Sohm et al. 2011, Knapp 2012, Cardini et al. 2014). However, thus far the effects of environmental variables on $N_2$ fixation have mostly been studied for free-living diazotrophs, while $N_2$ fixation associated with living corals has received much less attention. Lesser et al. (2007) found that $N_2$ fixation activity in the scleractinian coral *Monastraea cavernosa* follows a diurnal pattern, with maximum rates during twilight, and Davey et al. (2008) reported no effect of seasonal water temperature changes (22 vs. 28°C) on $N_2$ fixation associated with *Acropora aspera*. The coral reefs in the Gulf of Aqaba (northern Red Sea) represent a natural laboratory for studying the effect of seasonally changing environmental conditions on coral physiology. Their high-latitude location and the annual stratification cycle of the water column result in pronounced seasonal fluctuations in water temperature, light and inorganic nutrient availability (Silverman et al. 2007, Carlson et al. 2014).

In this study, we thus investigated $N_2$ fixation rates associated with 2 of the most dominant zooxanthellate soft corals, Xeniidae and *Sarcophyton* sp. (family: Alcyoniidae), from a fringing reef of the northern Gulf of Aqaba (Red Sea). Furthermore, responses of $N_2$ fixation, gross photosynthesis ($P_{gross}$) and dark respiration ($R$) rates (in terms of $O_2$ fluxes) to seasonally changing environmental conditions (e.g. inorganic nutrient concentrations, light intensity, water temperature) were studied over all 4 seasons within the year 2013 to identify how these 2 soft corals react to varying environmental conditions. Lastly, $N_2$ fixation was related to the $P_{gross}$ and $R$ rates of the corals to detect a potential linkage between diazotrophic activity and the physiology of the corals.

## MATERIALS AND METHODS

### Description of study site

This study was carried out during 2 expeditions (January to April and August to December 2013) to the Marine Science Station (MSS) at the northern Gulf of Aqaba, Jordan (29° 27′ N, 34° 58′ E). The MSS is situated at the Jordanian Red Sea coast approximately 10 km south of Aqaba city, with access to a fringing coral reef inside a marine reserve. To study the effect of seasonally changing environmental conditions on soft coral physiology, all experiments described below were conducted once in each of the months to represent all 4 seasons during the year 2013: February (winter), April (spring), September (summer), and November (fall).

### Soft coral distribution

Line point intercept (LPI) transects were carried out to determine relative soft coral cover in the study area. Three replicate 50 m LPI transects were conducted during each season at 1, 5, 10 and 20 m water depth. Benthic cover was recorded at 0.5 m intervals directly below the transect line (101 recorded data points per LPI transect). The relative abundance of soft corals was calculated as their percentage of benthic coverage.

### Soft coral collection and maintenance

Individual colonies of the genus *Sarcophyton* sp. (n = 8, average polyp number = 459 ± 41) and the family Xeniidae (n = 8, average polyp number = 64 ± 4) were collected during each season from the reef slope at 10 m water depth using SCUBA. To prevent any tissue damage, all soft corals were collected along with a small piece of the anchoring rock (<0.5 cm diameter) to which they were attached using a hammer and chisel. Subsequently, individual coral colonies, with their attachment rock, were fixed onto ceramic tiles using a 2-part epoxy putty (Reef Construct, Aqua Medic). The putty was used to cover the anchoring rock to ensure that encrusting communities on the rock did not affect rate measurements during subsequent incubations. All corals were transferred to an outdoor 800 l flow-through aquarium supplied with seawater pumped directly from the reef at 10 m water depth (exchange rate: 4000 l $h^{-1}$), thereby providing *in situ* water temperature and nutrient levels. Layers of netting were positioned above the tank to adjust light levels to those measured *in situ* at 10 m water depth with data loggers (HOBO Pendant UA-002-64, spectral detection range: 150 to 1200 nm, temperature accuracy: ±0.53°C, Onset). The corals were allowed to acclimate for 1 wk before further experimentation. All incubations took place in the outdoor 800 l flow-through aquarium to ensure the same water temperature, nutrient and light conditions and to avoid any stress to the coral colonies.

### Quantification of $O_2$ fluxes

The tiles with the attached corals were carefully cleaned of algal turf using a toothbrush. Each coral colony (n = 8) was transferred, without exposure to air, to individual 1 l glass chambers. In addition, 8 chambers filled only with seawater served as controls to measure planktonic background metabolism. The start $O_2$ concentration in each chamber was measured using a salinity-corrected $O_2$ optode sensor (FDO®925 Optical Dissolved Oxygen Sensor, range: 0.00 to 20.00 mg $O_2$ $l^{-1}$, accuracy: ±0.5% of the value, MultiLine® IDS 3430, WTW). All chambers were sealed gas tight (without any air bubbles inside) and incubated twice with constant stirring (600 rpm) on magnetic stirring plates for 1 to 2 h (Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific). After that, each chamber was opened to measure end $O_2$ concentrations. The first incubation was carried out 1 to 2 h after sunset to measure $R$ in complete darkness, while the second incubation started the following day between 12:00 and 13:00 h to determine net photosynthesis ($P_{net}$). To calculate $O_2$ fluxes ($P_{net}$ and $R$) from dark and light incubations, $O_2$ start concentrations were subtracted from end concentrations, and the results were normalized by incubation time. Finally, $O_2$ fluxes were corrected for the seawater control signal, related to incubation volume and normalized to the coral surface area. $P_{gross}$ rates were subsequently calculated according to $P_{gross} = P_{net} + R$. As $P_{net}$ was measured during the highest daily irradiance levels, and $R$ rates were shown to be significantly lower than light respiration rates for corals during active photosynthesis (Fabricius & Klumpp 1995, Al-Horani et al. 2003), the presented $P_{gross}$ rates are conservative estimates of daily maximum $O_2$ production.

### Quantification of $N_2$ fixation

$N_2$ fixation rates of the same soft coral colonies were measured 3 to 4 h after the light incubation for quantification of $P_{net}$ ended. An adapted acetylene ($C_2H_2$) reduction assay (ARA) was applied, as it was recently confirmed to be applicable for $N_2$ fixation quantification in oligotrophic waters (Capone 1993, Wilson et al. 2012). $C_2H_2$ gas was freshly generated from calcium carbide and bubbled through seawater to produce $C_2H_2$-enriched seawater. Without air exposure, each coral colony (n = 8) was placed individually in a 1 l glass chamber containing 800 ml of unfiltered seawater and 200 ml of air headspace. Then, 10% of the seawater (80 ml) was replaced with $C_2H_2$-enriched seawater before the chambers were closed gas tight. Immediately after, 10% of the headspace (20 ml) was replaced by $C_2H_2$ gas. The addition of $C_2H_2$ to the seawater minimizes the lag phase of the ARA because of a faster equilibration of $C_2H_2$ between the gas and liquid phase and an

immediate $C_2H_2$ saturation of the nitrogenase enzyme. In addition, 4 sets of controls were also tested for ethylene ($C_2H_4$) production form $C_2H_2$ reduction: (1) unfiltered seawater control (without coral fragments, n = 8); (2) 0.2 µm filtered seawater control (without coral fragments, n = 6); (3) tiles (without coral fragments) in unfiltered seawater (n = 6); and (4) coral fragments in unfiltered seawater without $C_2H_2$ addition (natural $C_2H_4$ production, n = 6). Over the whole incubation period (24 h), all chambers were constantly stirred (600 rpm), and gas samples were taken at the incubation start and after 4, 12, 16 and 24 h. At each of these time intervals, 1 ml of gas sample was collected with a gastight syringe from each chamber, transferred into gastight 2 ml vials previously filled with distilled water and stored frozen and upside down until analysis. $C_2H_4$ concentrations in the gas samples were measured in the field laboratory using a customized reducing compound photometer (RCP) (Peak Laboratories) with a detection limit of 100 ppb. The higher sensitivity of the RCP compared to the commonly used gas chromatograph equipped with a flame ionization detector allows best accurate estimations of $C_2H_4$ production rates. Calibration of the RCP was conducted using serial dilutions of a 200 ± 4 ppm $C_2H_4$ standard in air (Restek). To calculate $C_2H_4$ production rates of the coral fragments, $C_2H_4$ signals of the biological samples were corrected for seawater control signals (blank/biological ratios: 0.15 to 0.57) and subsequently normalized to incubation time and coral surface area. All rates are reported as nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$, since no parallel $^{15}N$ incubations were conducted, and the use of a theoretical conversion factor is controversial, as the $^{15}N$ method may have largely underestimated $N_2$ fixation until recently (Mohr et al. 2010). Additionally, it allows best comparability with the current literature, as the $C_2H_2$ reduction method has been most widely applied for benthic $N_2$ fixation quantification measurements in coral reefs (see Table 6).

### Surface area determination of soft corals

Activity rates ($N_2$ fixation, $P_{gross}$ and $R$) for each colony were related to coral surface area that was quantified using an advanced geometry approach (Naumann et al. 2009). This approach has already been applied for soft corals by Bednarz et al. (2012) and has been commonly used in physiological coral reef studies, thereby allowing best comparability of the present data to previous studies. Briefly, the number of polyps of each coral colony was counted, and each of these polyps was mathematically handled as the area of a circle. The surface area of 50 completely expanded polyps randomly distributed over all colonies was measured separately for Xeniidae and *Sarcophyton* sp. using the image analysis software ImageJ (National Institutes of Health, USA). Subsequently, the average circular surface area of a polyp was calculated ($r^2 \times \pi$, where $r$ = radius) and multiplied by the number of polyps per colony. In addition, the surface area of the body foot of each colony was approximated to a cylinder ($2 \times \pi \times r \times h$, where h = height) by measuring its diameter ($2 \times r$) and height using a caliper (accuracy ±0.01 cm). To generate the total surface area of each incubated Xeniidae and *Sarcophyton* sp. colony, the total number of polyps of each colony was multiplied with the average surface area of an expanded polyp and subsequently added to the surface area of the body foot.

### Monitoring of environmental parameters

During each season, water temperature and light intensity (lux) at 10 m water depth were continuously recorded over 4 wk by data loggers (HOBO Pendant UA-002-64, temperature accuracy: ±0.53°C, spectral detection range: 150 to 1200 nm, Onset). The presented light intensities were standardized to the time of day with maximum light intensities (11:00 to 13:00 h), and lux readings were converted to photosynthetically active radiation (PAR, µmol quanta $m^{-2}$ $s^{-1}$, wavelength 400 to 700 nm) using the following approximation: 1 µmol quanta $m^{-2}$ $s^{-1}$ = 52.0 lux. This conversion factor was obtained by intercalibrating the lux readings with data obtained from a PAR sensor (LI-COR LI-192SA underwater quantum sensor) during a simultaneous minute-by-minute measurement over 5 h. Both readings correlated well ($r^2$ = 0.83), and the obtained conversion factor of 52.0 was very similar to 51.2 reported by Valiela (1984). Once a week, seawater samples (50 ml, n = 4) were taken from the place of coral collection. After filtering the seawater through sample-washed cellulose acetate membrane filters (nominal pore size: 0.45 µm), inorganic nutrient (ammonium, phosphate) concentrations were immediately measured following methods described by Holmes et al. (1999) and Murphy & Riley (1962). Ammonium was determined fluorimetrically using a Trilogy fluorometer (Turner Designs) with a detection limit of 0.09 µM, while phosphate was measured photometrically with a JASCO-V630 spectrophotometer and a detection limit of 0.01 µM.

### Statistical analysis

All statistical analyses were carried out using PRIMER-E version 6 software (Clarke & Gorley 2006) with the PERMANOVA+ add-on (Anderson 2001). Analyses were based on Euclidean distance of environmental data (normalized) and Bray-Curtis similarities of physiological parameters (square root transformed). A principal coordinate analysis and a 1-factor PERMANOVA with type III (partial) sum of squares and unrestricted permutation of raw data (999 permutations) was used to test for seasonal differences of the environmental variables (water temperature, light intensity, inorganic nutrients; colinear variables ammonium and phosphate are summarized). Two-factor PERMANOVAs were performed to test for differences of physiological parameters ($N_2$ fixation, $P_{gross}$, $R$) between soft corals and seasons. Therefore, type I (sequential) sum of squares was used with permutation of residuals under a reduced model (999 permutations), and pairwise tests were carried out when significant differences occurred. In addition, a nonmetric multidimensional scaling ordination of the physiological parameters ($N_2$ fixation, $P_{gross}$ and $R$) in relation to soft coral and season was conducted. Finally, correlations between the physiological variables as well as between the physiological variables and the environmental factors were determined via linear regression.

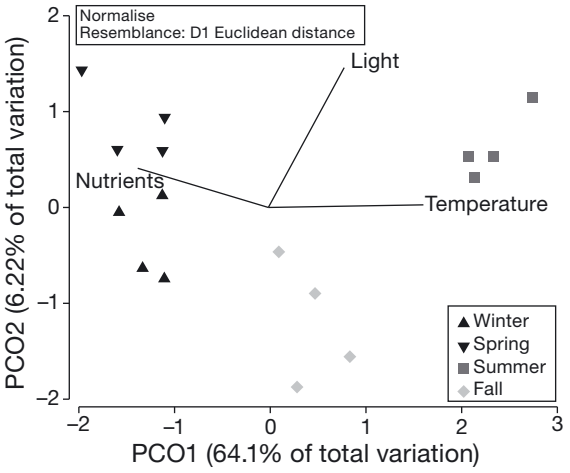


Fig. 1. Principal coordinate analysis performed on Euclidean distance matrix and normalized data for the different environmental variables (temperature, light, inorganic nutrients) measured in seawater samples from 10 m water depth once a week during 4 different seasons (winter, spring, summer, fall). PCO1: first principal coordinate, PCO2: second principal coordinate

## RESULTS

### Environmental conditions and soft coral cover

The 4 seasons differed significantly from each other with respect to the environmental water parameters (PERMANOVA, df = 3, SS = 51.138, MS = 17.046, pseudo-$F$ = 23.083, p = 0.001). The winter and spring season were most similar to each other, followed by fall, with lower inorganic nutrient concentrations and higher water temperatures. The summer season was most distinct from the other 3 seasons and exhibited the highest water temperatures and light intensities but the lowest inorganic nutrient concentrations (Fig. 1, Table 1).

Despite these strong seasonal changes in environmental water parameters, the soft coral cover remained constant throughout the year at each water depth (Table 2). Over all depths, soft coral cover was approximately 8.6 ± 0.8%, whereby the highest cover was recorded at 10 m water depth with a percentage cover of 21.6 ± 1.3%. Most of the soft coral cover was represented by Xeniidae that was approximately 10 times more abundant than *Sarcophyton* sp.

### $N_2$ fixation and $O_2$ fluxes

Active $N_2$ fixation rates occurred throughout the year for both soft corals, indicated by the up to 75-fold higher $C_2H_4$ production in coral-containing incubation chambers compared to the seawater controls (Table 3). Soft coral-associated $N_2$ fixation revealed

Table 1. Summary of environmental water parameters monitored over 4 wk during each of the 4 different seasons at 10 m water depth. During each season, water temperature was continuously recorded and is averaged over the 4 wk period, while light intensity represents maximum values measured daily between 11:00 and 13:00 h. Ammonium and phosphate concentrations were measured once a week from seawater samples (n = 4) taken in the early morning. Values are means, with SE in parentheses. PAR: photosynthetically active radiation (µmol quanta $m^{-2}$ $s^{-1}$)

| Month (season) | Temperature (°C) | Light intensity (PAR) | Ammonium (µM) | Phosphate (µM) |
|---|---|---|---|---|
| Jan/Feb (winter) | 22.97 (0.11) | 180 (15) | 0.32 (0.04) | 0.11 (0.01) |
| Mar/Apr (spring) | 22.78 (0.10) | 257 (9) | 0.46 (0.03) | 0.10 (0.01) |
| Sep (summer) | 27.52 (0.16) | 317 (17) | 0.14 (0.03) | 0.04 (0.01) |
| Nov (fall) | 25.19 (0.17) | 159 (18) | 0.28 (0.06) | 0.04 (0.01) |

Table 2. Benthic soft coral cover in different water depths and relative contribution by *Sarcophyton* sp., Xeniidae and other soft corals. Values are annual means (n = 12), with SE in parentheses

| Depth (m) | Benthic soft coral cover (%) | Soft coral composition (%) | | |
|---|---|---|---|---|
| | | *Sarcophyton* sp. | Xeniidae | Others |
| 1 | 3.2 (0.6) | 10.9 (8.7) | 2.3 (2.2) | 86.8 (8.7) |
| 5 | 3.0 (0.7) | 3.2 (1.9) | 57.4 (12.1) | 45.4 (11.9) |
| 10 | 21.6 (1.3) | 5.4 (2.3) | 91.0 (2.2) | 3.5 (1.3) |
| 20 | 6.6 (0.6) | 6.7 (2.7 | 88.8 (3.5) | 4.5 (2.3) |
| Average | 8.6 (0.8) | 6.6 (3.9) | 59.9 (5.0) | 35.1 (6.1) |

Table 3. Dinitrogen ($N_2$) fixation (nmol $C_2H_4$ $l^{-1}$ $h^{-1}$) measured in soft coral and seawater control (without corals) incubation chambers. Values are normalized to 1 l incubation water and presented as means of n = 8 replicates, with SE in parentheses

| Incubation | Winter | Spring | Summer | Fall |
|---|---|---|---|---|
| *Sarcophyton* sp. | 2.07 (0.83) | 4.05 (0.92) | 14.26 (1.77) | 3.57 (1.47) |
| Xeniidae | 1.20 (0.25) | 0.68 (0.08) | 2.13 (0.57) | 0.61 (0.12) |
| Seawater control | 0.06 (0.04) | 0.36 (0.05) | 0.19 (0.06) | 0.15 (0.08) |

significant effects of soft coral, season and their interaction (Fig. 2, Table 4). The corals exhibited similar rates during the winter season, but *Sarcophyton* sp. showed significantly higher rates in the other 3 seasons compared to Xeniidae. Rates ranged during the year from 0.004 to 0.205 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$ for *Sarcophyton* sp. and from 0.001 to 0.096 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$ for Xeniidae, with annual averages of 0.055 ± 0.011 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$ and 0.019 ± 0.005 nmol $N_2$ $cm^{-2}$ $h^{-1}$, respectively. Besides these soft coral-specific differences, both soft corals showed the same seasonal pattern of $N_2$ fixation activity. While no differences occurred between winter, spring and fall, $N_2$ fixation rates significantly increased during the summer season for both investigated soft corals. Overall, summer rates were 3 to 6 (*Sarcophyton* sp.) and 6 to 14 times (Xeniidae) higher than $N_2$ fixation during the other seasons.

$O_2$ fluxes ($P_{gross}$ and $R$) also exhibited soft coral-specific and seasonal differences (Fig. 2, Table 4). Averaged among all seasons, Xeniidae (15.6 ± 0.8 µg $O_2$ $cm^{-2}$ $h^{-1}$) revealed significantly higher $P_{gross}$ rates, approximately 1.5-fold higher than *Sarcophyton* sp. (11.7 ± 0.8 µg $O_2$ $cm^{-2}$ $h^{-1}$). On a seasonal scale, Xeniidae displayed the highest $P_{gross}$ rates during spring, while *Sarcophyton* sp. had maximum rates during both spring and summer. In contrast, $R$ rates were constantly lower in Xeniidae (2.8 ± 0.2 µg $O_2$ $cm^{-2}$ $h^{-1}$) compared to *Sarcophyton* sp. (4.9 ± 0.3 µg $O_2$ $cm^{-2}$ $h^{-1}$) averaged among all seasons. Xeniidae exhibited highest $R$ rates in both spring and summer, while $R$ rates of *Sarcophyton* sp. peaked during the summer season, thereby following the seasonal pattern of $N_2$ fixation rates.

## Relationships between $N_2$ fixation, $O_2$ fluxes and environmental factors

$N_2$ fixation and $O_2$ fluxes ($P_{gross}$ and $R$) clearly showed a separation between the 2 soft corals as well as a distinct difference in summer compared to the other 3 seasons (Fig. 3). Separation of summer from the other seasons was mainly driven by changes in $N_2$ fixation and $R$ rather than by changes in $P_{gross}$, suggesting a potential linkage between $N_2$ fixation and $R$. Indeed, linear regression analysis revealed a significant positive relationship between $R$ and $N_2$ fixation for *Sarcophyton* sp. ($F$ = 16.070, $r^2$ = 0.373, $p < 0.001$) but not for Xeniidae ($F$ = 1.249, $r^2$ = 0.043, $p$ = 0.273). In contrast, no significant relationship between $P_{gross}$ and $N_2$ fixation was found, neither for *Sarcophyton* sp. ($F$ = 2.784, $r^2$ = 0.093, $p$ = 0.107) nor for Xeniidae ($F$ = 0.047, $r^2$ = 0.002, $p$ = 0.8297).

$N_2$ fixation of both soft corals showed similar relationships with each water parameter, with negative

Table 4. Results of 2-factorial PERMANOVAs for dinitrogen ($N_2$) fixation (nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$), gross photosynthesis ($P_{gross}$; µg $O_2$ $cm^{-2}$ $h^{-1}$) and dark respiration ($R$; µg $O_2$ $cm^{-2}$ $h^{-1}$) rates for 2 soft corals (*Sarcophyton* sp. and Xeniidae) and 4 different seasons (winter, spring, summer, fall). Soft coral (Sc) and season (Se) were fixed effects. PERMANOVA was based on Bray-Curtis similarity after square root transformation. Type 1 (sequential) sum of squares was used with permutation of residuals under a reduced model (999 permutations). Significant p values are in **bold**

| Variable | Effect | df | SS | MS | Pseudo-$F$ | p |
|---|---|---|---|---|---|---|
| $N_2$ fixation | Sc | 1 | 10144 | 10144 | 31.34 | **0.001** |
| | Se | 3 | 19555 | 6519 | 20.14 | **0.001** |
| | Sc × Se | 3 | 5208 | 1736 | 5.36 | **0.001** |
| $P_{gross}$ | Sc | 1 | 918 | 918 | 16.66 | **0.002** |
| | Se | 3 | 1350 | 450 | 8.17 | **0.001** |
| | Sc × Se | 3 | 129 | 43 | 0.78 | 0.513 |
| $R$ | Sc | 1 | 2534 | 2534 | 77.52 | **0.001** |
| | Se | 3 | 1503 | 501 | 15.33 | **0.001** |
| | Sc × Se | 3 | 332 | 111 | 3.38 | **0.032** |

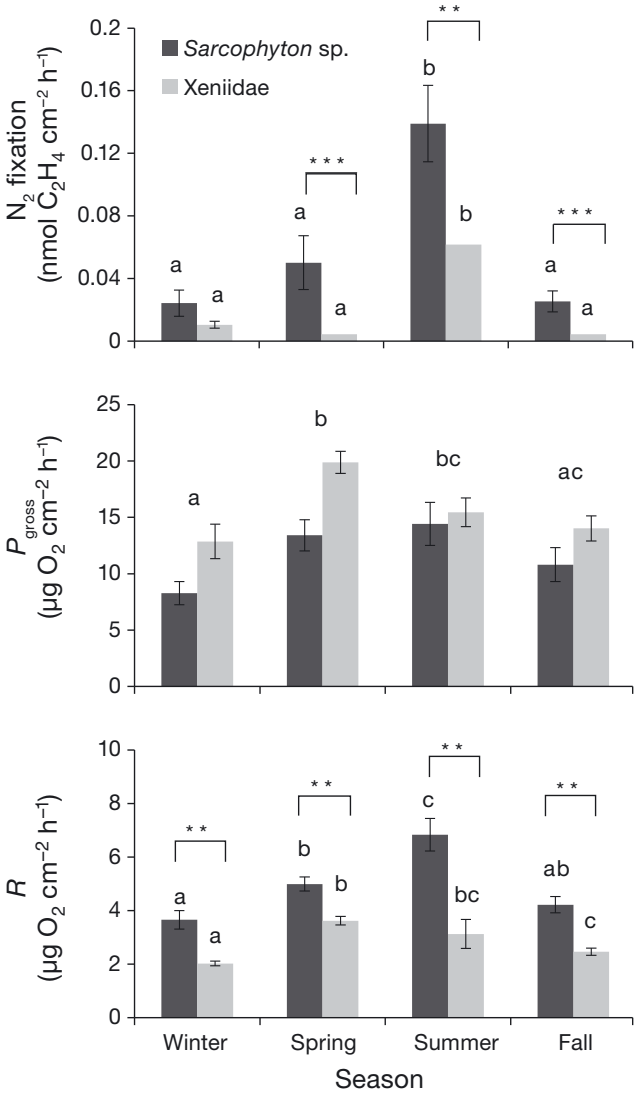


Fig. 2. Rates of dinitrogen ($N_2$) fixation, gross photosynthesis ($P_{gross}$) and dark respiration ($R_{dark}$) for *Sarcophyton* sp. and Xeniidae measured during 4 different seasons (winter, spring, summer, fall) in 2013. Values are given as mean ± SE (n = 8). Asterisks indicate significant differences between the 2 soft corals during each season (**p < 0.005, ***p < 0.001); different letters indicate significant differences between the seasons for *Sarcophyton* sp. and Xeniidae, respectively, based on pair-wise PERMANOVA analysis

correlations to ammonium and phosphate concentrations and positive correlations to water temperature and light intensity (Table 5). *R* rates of *Sarcophyton* sp. showed the same correlation to the water parameters as $N_2$ fixation. In contrast, no correlations between *R* rates of Xeniidae and any water parameters were found. $P_{gross}$ of Xeniidae was positively correlated to ammonium concentration, while $P_{gross}$ of *Sarcophyton* sp. was positively correlated to light intensity.

Table 5. Linear regression analysis ($r^2$ values) between dinitrogen ($N_2$) fixation (nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$), gross photosynthesis ($P_{gross}$; µg $O_2$ $cm^{-2}$ $h^{-1}$) and dark respiration (*R*; µg $O_2$ $cm^{-2}$ $h^{-1}$) rates of 2 soft corals (*Sarcophyton* sp. and Xeniidae) and 4 different environmental water parameters (ammonium concentration, phosphate concentration, water temperature and light intensity). **Bold** characters indicate significant positive relationships, and *italicized* characters indicate significant negative relationships (*p < 0.05, **p < 0.005, ***p < 0.001)

| | Ammonium | Phosphate | Water temp. | Light intensity |
|---|---|---|---|---|
| *Sarcophyton* sp. | | | | |
| $N_2$ fixation | *0.217** | *0.137** | **0.344***** | **0.478***** |
| $P_{gross}$ | 0.001 | 0.047 | 0.060 | **0.180*** |
| *R* | *0.142** | *0.158** | **0.326**** | |
| Xeniidae | | | | |
| $N_2$ fixation | *0.561***** | *0.222**** | **0.592***** | **0.513***** |
| $P_{gross}$ | **0.144*** | 0.022 | 0.021 | 0.130 |
| *R* | 0.040 | 0.000 | 0.002 | |

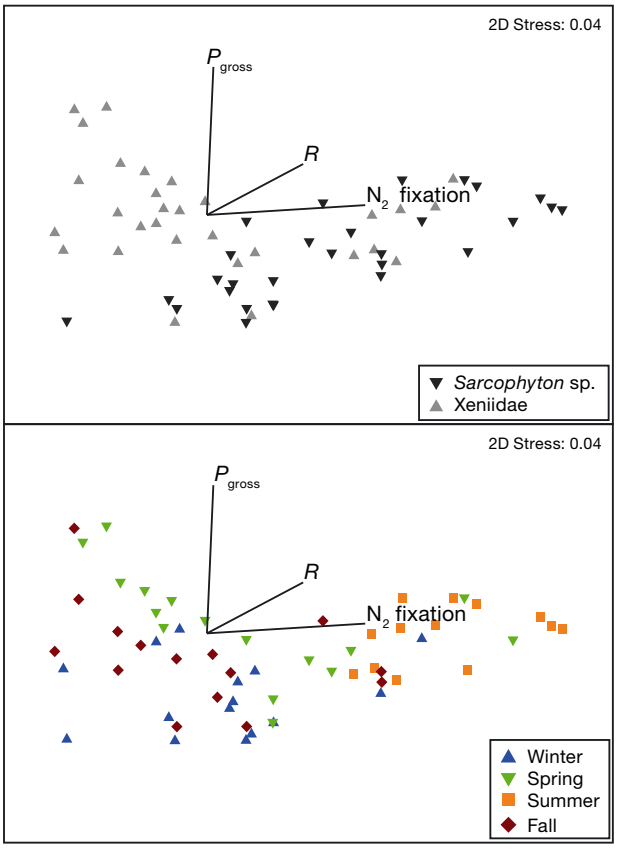


Fig. 3. Multidimensional scaling plot of dinitrogen ($N_2$) fixation, gross photosynthesis ($P_{gross}$) and respiration (*R*) rates for 2 soft corals (*Sarcophyton* sp. and Xeniidae) and 4 different seasons (winter, spring, summer, fall). Analysis was performed on Bray-Curtis similarities of square root-transformed data

## DISCUSSION

### Soft coral-specific $N_2$ fixation and $O_2$ fluxes

This study for the first time identified $N_2$ fixation rates associated with 2 dominant zooxanthellate soft corals from an oligotrophic, subtropical coral reef environment. Previous studies, mainly focused on hard corals, identified their different hard coral-associated $N_2$-fixing bacteria (Rohwer et al. 2002, Wegley et al. 2007, Olson et al. 2009, Lema et al. 2012) or quantified their $N_2$ fixation rates (Shashar et al. 1994a, Davey et al. 2008, Lesser et al. 2007). These rates are 1 to 2 orders of magnitude higher than the rates detected for *Sarcophyton* sp. and Xeniidae in the present study (Table 6). These apparent differences in $N_2$ fixation rates may be due to abundance and composition differences of the diazotrophic communities associated with hard and soft corals. Lema et al. (2012) demonstrated that hard coral species form specific associations with diazotrophs which may further result in species-specific $N_2$-fixing activities. Likely, hard and soft corals may harbor a very distinct diazotrophic community because of the presence of endolithic algae and endolithic bacteria in the former. Endolithic, heterotrophic bacteria are most likely responsible for the majority of $N_2$ fixation activity in scleractinian corals (Shashar et al. 1994a), which was supported by measurements of endolithic ammonium excretion rates (0.8 to 1.4 nmol $NH_4^+$ $cm^{-2}$ $h^{-1}$) that matched the rates of $N_2$ fixation (Ferrer & Szmant 1988). The main energy source for $N_2$ fixation is likely provided via the excretion of organic photosynthates by the coral into the coralline skeleton, thereby establishing a suitable microhabitat for a diazotrophic community that is absent in soft corals. Therefore, soft corals may harbor fewer diazotrophs compared to scleractinian corals that may explain their lower $N_2$ fixation rates.

So far, $N_2$ fixation has only been investigated for 2 octocoral species (*Tubipora musica* and *Rhytisma fulvum*) using the ARA (Shashar et al. 1994b). Those authors measured rates of 35.7 ± 14.2 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$, thus exceeding rates quantified in the present study for *Sarcophyton* sp. and Xeniidae by 2 to 3 orders of magnitude. The octocoral species *T. musica* contains an endoskeleton-providing habitat for endolithic diazotrophs comparable to scleractinian corals, while *R. fulvum* is an azooxanthellate, encrusting soft coral species. Because of its encrusting morphology, *R. fulvum* is extremely difficult to separate from its attaching substrate, and Shashar et al. (1994b) did not remove the epilithic algae from soft coral. This may result in biased $N_2$ fixation rates, as the activity of diazotrophs which are not associated with the coral is also taken into account. Shashar et al. (1994b) also present $N_2$ fixation rates of other unidentified cnidarians and sponges, which are similar to the rates from the present study. Overall, this demonstrates that there is a wide range of

Table 6. Reported dinitrogen ($N_2$) fixation rates (nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$) for benthic reef organisms and substrates in comparison with annual averages of the soft corals *Sarcophyton* sp. and Xeniidae from the present study

| Organism/substrate | $N_2$ fixation (nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$) | Location | Reference |
|---|---|---|---|
| Octocorals | | | |
| Xeniidae | 0.001–0.096 | Aqaba, Red Sea | This study |
| *Sarcophyton* sp. | 0.004–0.205 | Aqaba, Red Sea | This study |
| *Tubipora musica* and *Rhytisma fulvum* | 35.7 ± 14.2 | Eilat, Red Sea | Shashar et al. (1994b) |
| Scleractinian corals | | | |
| *Acropora aspera* | 0.56–1.16 | Australia, Great Barrier Reef | Davey et al. (2008) |
| *Acropora* sp. | 8.7 ± 7.3 | Eilat, Red Sea | Shashar et al. (1994a) |
| *Stylophora pistillata* | 6.4 ± 2.4 | Eilat, Red Sea | Shashar et al. (1994a) |
| *Pocillopora damicornis* | 0.6 ± 0.4 | Eilat, Red Sea | Shashar et al. (1994a) |
| Other cnidarians | 0.1 ± 0.3 | Eilat, Red Sea | Shashar et al. (1994b) |
| Sponges | 0.2 ± 0.4 | Eilat, Red Sea | Shashar et al. (1994b) |
| Dead coral skeleton | 55.45 ± 28.5 | Eilat, Red Sea | Shashar et al. (1994b) |
| Dead coral skeleton | 0.15–12.77 | Australia, Great Barrier Reef | Davey et al. (2008) |
| Algal substrate | 9.25 ± 0.5 | Eilat, Red Sea | Shashar et al. (1994b) |
| Microbial mats | 0.59–2.97 | Indian Ocean | Charpy et al. (2012) |
| Carbonate sand | 0.18 ± 1.02 | French Polynesia | Charpy-Roubaud et al. (2001) |
| Carbonate sand | 19.5 ± 17.5 | Eilat, Red Sea | Shashar et al. (1994b) |

$N_2$ fixation activity among different benthic coral reef organisms.

Although $N_2$ fixation rates associated with *Sarcophyton* sp. and Xeniidae are in the lower range among benthic reef organisms, the rates are up to 75-fold higher compared to $N_2$ fixation in the seawater controls, thus confirming that the 2 soft corals are associated with active $N_2$-fixing bacteria. $N_2$ fixation in the seawater controls (0.8 to 4.3 nmol N $l^{-1}$ $d^{-1}$, obtained using 4:1 as the $C_2H_4$:$N_2$ conversion factor) is slightly higher but within the range of seawater $N_2$ fixation previously measured in the Gulf of Aqaba using the $^{15}N_2$ method (0.1 to 1.9 nmol N $l^{-1}$ $d^{-1}$; Foster et al. 2009). The classical $^{15}N_2$ method underestimates $N_2$ fixation rates (Mohr et al. 2010) when the $^{15}N_2$ tracer is only introduced as a gas bubble, as in Foster et al. (2009), while the addition of $^{15}N_2$-enriched seawater accelerates the gas equilibration process and improves the accuracy of the method (Wilson et al. 2012). Similarly, the ARA can reliably quantify $N_2$ fixation in oligotrophic waters if $C_2H_2$-enriched seawater is used, as in the present study (Wilson et al. 2012). These methodological differences provide reasoning for the slightly higher rates obtained in our seawater controls and confirm the validity of the soft coral-associated $N_2$ fixation rates.

The 2 zooxanthellate soft corals investigated in the present study also revealed differences in their specific $N_2$ fixation activity, with significantly higher rates in *Sarcophyton* sp. compared to Xeniidae on an annual average. Lower $N_2$ fixation rates in Xeniidae may be caused by highly oxygenated areas due to higher $P_{gross}$ and lower $R$ rates, which indicate a more autotrophic nutrition by Xeniidae compared to *Sarcophyton* sp. This is confirmed by Schlichter et al. (1983), who characterized soft corals of the family Xeniidae as functional autotrophic plant animals. Also, compared to *Sarcophyton* sp., Xeniidae exhibit nonretractile, pumping polyps, thereby creating conditions which facilitate photosynthesis. Pumping increases water exchange between the boundary layer of the organism and the water body (Mass et al. 2010, Kremien et al. 2013), while polyp expansion increases the surface area for potential gas exchange through the epidermal tissue (Fabricius & Klumpp 1995). Furthermore, the polyp's tip hosts most of the organism's zooxanthellae and represents the photosynthetically most active tissue. Polyp retraction reduces light exposure for zooxanthellae and can decrease photosynthesis in several soft corals (Fabricius & Klumpp 1995). This may help explain the higher photosynthesis rates measured in Xeniidae during each season, which likely leads to hyperoxic conditions in the tissue during daytime (Shashar et al. 1993, Kühl et al. 1995). As the nitrogenase enzyme is highly sensitive to $O_2$ (Postgate 1982), it may explain lower $N_2$ fixation rates in Xeniidae compared to *Sarcophyton* sp.

### Seasonal variation in $N_2$ fixation and $O_2$ fluxes

In the present study, we measured for the first time the effect of seasonally changing environmental conditions on $N_2$ fixation associated with soft corals. Both of the 2 investigated soft corals showed the highest $N_2$ fixation rates during summer, when ammonium availability in the ambient seawater was low, but water temperature and irradiance were high. This is supported by the linear regression analysis revealing for $N_2$ fixation a significant negative relationship to inorganic nutrients and positive correlations to water temperature and light intensity (Table 5). This seasonal pattern is in accordance with 2 recent studies on pelagic diazotrophs in the Gulf of Aqaba (Foster et al. 2009, Rahav et al. 2013). Both measured up to 6 times higher $N_2$ fixation rates in the photic water layer during the stratified summer and early fall months compared to the well-mixed conditions in winter and spring. High water temperature can stimulate the enzymatic activity of nitrogenase (Capone et al. 1997, 2005), while high ammonium concentrations in the water can clearly inhibit $N_2$ fixation, as demonstrated in laboratory studies on diazotroph cultures (reviewed in Sohm et al. 2011). Roughly 25% more energy is required to reduce $N_2$ (87 kcal) than $NO_3^-$ (69 kcal) to ammonium; therefore, it is energetically inefficient to fix $N_2$ in marine environments with DIN concentrations above a certain threshold (~1 µM). High irradiance can inhibit the process of $N_2$ fixation indirectly because of enhanced photosynthetic $O_2$ production. However, $P_{gross}$ of the 2 investigated soft corals showed only a slight increase during summer, which started already during spring, when inorganic nutrient availability in the water column was highest. Thus, the strong increase in $N_2$ fixation during summer is most likely caused by a combination of these different environmental factors but may be more strongly influenced by the direct effects of high water temperature and low nutrient availability than by the indirect effects of light.

Corals have developed several seasonal adaptations to protect the photosystem from harmful photons during the summer months. This includes, for

example, the down-regulation of photosynthesis by the zooxanthellae (Warner et al. 2002), the decrease of zooxanthellae abundance (Fitt et al. 2000) or the enhanced production of photoprotecting mycosporine-like amino acids (MAAs) (Michalek-Wagner 2001). The present study thus suggests that the high $N_2$ fixation during summer may provide soft corals with the N compounds needed to produce MAAs during the brightest periods of the year or to regain their zooxanthellae density during the following recovery phase.

In both soft corals, *R* revealed a trend similar to $N_2$ fixation, with maximum rates during summer. This is also supported by the positive relationship between $N_2$ fixation and *R* rates, suggesting linkage between both processes. During summer, the respiratory metabolism of corals generally increases as they build up biomass for reproduction (Shlesinger et al. 1998, Fitt et al. 2000). For example, in the soft coral *Heteroxenia fuscescens* (Xeniidae) from the northern Red Sea, the biochemical tissue composition changed over the year, with the highest energy content during summer, followed by spring, fall and finally winter (Ben-David-Zaslow & Benayahu 1999). This seasonal pattern reflects the high coral fecundity and reproduction in summer (Ben-David-Zaslow et al. 1999). Thus, reproduction during summer may be supported by the increased availability, and use, of $N_2$ fixation products. Given the low DIN availability and the low $N_2$ fixation activity in the water column of the Gulf of Aqaba (Foster et al. 2009, Rahav et al. 2013), as well as the importance of N for cell maintenance, growth and functioning, the association with $N_2$-fixing bacteria may be a key component of soft coral nutrition during summer.

## Ecological implications

Corals profit from the association with several symbionts including $N_2$-fixing bacteria. Although the present study could not determine whether the diazotrophs are internally (i.e. as endosymbionts in the coral tissue; Lesser et al. 2004) or externally associated with the coral (i.e. in the mucus layer; Lema et al. 2012), both associations are likely to benefit the coral holobiont by providing bioavailable N. The zooxanthellae may also internally harbor diazotrophs, thereby directly receiving fixed N, similar to what has been shown for a different diazotrophs–eukaryotic algae symbiosis (Foster et al. 2011). Overall, the ability of corals to acquire N both via diazotrophy and via uptake of DIN from the surrounding seawater is advantageous in an environment where the availability of dissolved nutrients is generally low and episodic. Recently, enzymes enabling ammonium assimilation were detected in endosymbiotic algae and the coral host, suggesting that both could benefit from the products of $N_2$ fixation (Leggat et al. 2007, Yellowlees et al. 2008, Stambler 2011). The present study suggests that uptake of DIN from the seawater may fuel coral metabolism during the nutrient-enriched spring period, while $N_2$ fixation products may be a key component of coral nutrition during the nutrient-depleted summer months.

Given the usual low rates of $N_2$ fixation in the water column of nutrient-poor coral reefs (Foster et al. 2009, Rahav et al. 2013), benthic reef organisms, including soft corals, may provide habitat for diazotrophs, thereby playing a key role for the input of new N into the reef ecosystem. In many reef locations worldwide, soft corals represent the second most dominant benthic group after scleractinian corals (Benayahu & Loya 1977, Fabricius & De'ath 2001, Inoue et al. 2013), and in the investigated study area, soft coral cover has increased by 50% since 2007 (C. Wild unpubl. data), now reaching up to 21.6% of the total benthic cover. Therefore, the present study suggests that soft corals may contribute importantly to the overall input of fixed N within the reef, although $N_2$ fixation rates per unit surface area are low compared to scleractinian corals. Soft corals also represent major space competitors for hard corals, and shifts from hard to soft coral dominance has been observed in several reef locations worldwide (Tilot et al. 2008, Norström et al. 2009). The data in the present manuscript thus indicate that input of new N via $N_2$ fixation may be reduced in soft coral reefs compared to those dominated by hard corals, with potential implications on biogeochemical element cycles and reef ecosystem functioning.

*Acknowledgements*. We thank L. Rix, M. S. Naumann, F. Al-Horani and S. Basuoni for fieldwork assistance and logistical support; M. Birkicht, D. Peterke and S. Wilson for their help with sample analysis; and J. G. Plass-Johnson for comments on the manuscript. This study was funded by German Research Foundation (DFG) grant Wi 2677/6-1 to C.W. and a PhD stipend from Evangelisches Studienwerk Villigst eV to V.N.B.

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*Editorial responsibility: Pei-Yuan Qian, Kowloon, Hong Kong SAR*

*Submitted: May 15, 2014; Accepted: October 22, 2014*
*Proofs received from author(s): December 25, 2014*