Water quality monitoring Bonaire

Identification of indicators, methods and locations

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Summary

On the island Bonaire, eutrophication is a point of serious concern, affecting the coral reefs in the marine park. Eutrophication can cause altered balance of the reef ecosystem because algae shall outcompete corals, eventually leading to a disturbed composition of the reef.
The reef of Bonaire faces nutrient input by various sources, of which enriched groundwater outflow from land to the reef is considered to be a substantial source. Groundwater is enriched with nutrients due to the e.g. leaking septic tanks.
In order to reduce the input of nutrients on the reef via sewage water, a water treatment plant is being built on Bonaire. The treatment of sewage water will be extended in 2012 with a sewage system covering the so called sensitive zone, the urbanised area from Hato to Punt Vierkant. Based on the dimensions of the treatment plant and estimated connections to the plant, it can be assumed that a total of 17520-35040 kg of Nitrogen a year will be removed from the sensitive zone, and will not leach out to the sea at the western coast of Bonaire.
At the moment limited information is available about the total amount of nutrients in the marine environment. Therefore, Rijkswaterstaat Waterdienst asked IMARES to conduct a monitoring study.The goal of this coastal monitoring study was to collect baseline water quality data to be able to study the effectiveness of the water treatment facility in coming years. No estimates are known of the contribution of other sources to the total nitrogen load.

The study consisted of two phases and resulted in two reports:

1. recommendations for baseline monitoring in 2011,
2. monitoring, data evaluation, and recommendations

The aim of this first report was to define recommendations for the baseline monitoring the, expected positive, impact of the new sewage treatment system on the marine environment of Bonaire, with special emphasis on baseline monitoring. For this an evaluation was made of

- Parameters/indicators to analyse, including argumentation, critical conditions
- Methods for sampling and critical conditions, including costs
- Potential sampling locations

Indicators and methods, baseline values
In this report, several indicators are described in context of the research question, available methods and costs, and critical conditions. Background variability, and environmental threshold levels are reported as well.

Based on previous environmental studies and on the relation to the treatment plant the following indicators were selected:
Nutrient concentrations: steer eutrophication, indicative for enriched groundwater. Volumes of polluted groundwater are expected to decrease as result of the installation of the water treatment plant. Nutrient levels are expected to decrease consequently. However, other (natural and human induced) sources can interfere, as well as the fact that nutrients are in a constant flux and expected to be (naturally) present in very low levels.
Stable isotope d15N: Wastewater nutrients from e.g. septic systems, and sewage treatment plants are generally enriched in the heavier nitrogen isotope, 15N. Algae are usually plentiful present in reef ecosystems and have been shown faithfully to track sewage input via 15N. Volumes of polluted groundwater are expected to decrease as result of the installation of the water treatment plant. 15N levels are expected to decrease consequently.

Chlorophyll a: Elevated nutrient levels steer primary production and thus algal growth, reflected by chlorphyll a.

Bacteria (enterococci): Septic tanks might leak or overflow into the groundwater, en enter the reef via the outflow. Enterococci are a good proxi for monitoring fecal bacteria. Volumes of polluted groundwater are expected to decrease as result of the installation of the water treatment plant. Bacteria levels are expected to decrease consequently.

General water parameters: help to evaluate data, and could indicate eutrophication in an indirect manner.

Benthic composition: Some bio indicators are indicative for nutrient stress, e.g. the abundance of bio eroders (sponges). These indicators are covered by annual monitoring by STINAPA and reported in separate reports. This type of monitoring is not conducted again under the term of this project.

Background values of nutrients in reef ecosystems are generally very low, and selection of laboratories was steered by the suitability of proper detection limits, and the available and trustworthy logistics. This means that due to risk of delayed transport to the US, these laboratories were not included in the final selection. In table I, an overview is provided per indicator, its relation to the treatment plant, the analysis, and environmental threshold values, if available.

Table I overview per indicator

	indicative for		**Analysis**		**environmental threshold**
Indicator	**Treatment plant**	**other pressures**	**Method**	**laboratory/ institute**	
General (Temperature, pH, dissolved oxygen, salinity, turbidity)	indirect	yes (biotic, abiotic)	multimeter	In situ	3 NTU
Nutrients (NH_4, NO_2, NO_3, PO_4)	Yes	yes (biotic, abiotic)	continuous flow analyser	NIOO	DIN: 1 µmol/L, P: 0.1 µmol/L
Chlorophyll a	indirect	yes (biotic, abiotic)	aceton extraction	IMARES	0.5 µg/L
Stable isotopes	Yes+	yes via foodweb	mass spectrometer	NIOZ	3 ‰
Bacteria (enterococci)	Yes	Yes	Enterolert IDEXX	CIEE	>185 cfu/100ml
Benthic composition	Yes	Yes	AGGRA	STINAPA, in prep	various

For each parameter, a field and lab protocol is established on how to perform sampling, and pre-process the sample in the laboratory before actual analysis. The specifications are included in the annexes of this report.

Furthermore, it is advised to make use of the facilities of CIEE Bonaire. This laboratory offers local and near site provisions needed to conduct the proposed monitoring in a proper manner.

An overview of analysis costs per indicator are included in the annex.

<u>Locations</u>

Location selection was based on several criteria, being geographical order, influence of sewage sources (via groundwater), influence of other factors, data availability from previous studies, serving as reference location.

A so-called “sensitive zone” is delineated extending 200 m inland from the coast, between Punt Vierkant and Hato. In this area, wastewater is produced in large quantities. The majority of this wastewater ends

up in the marine environment via outflow, run off, irrigation or percolation. Locations within the sensitive zone are included as being "worst case" locations. At these locations all indicators are expected to show the strongest response to the installation of the treatment plant.
Locations outside the sensitive zone can be regarded as 'relative" reference locations. Locations outside sensitive zone and downstream of the prevailing currents can be influenced by the salt company. Prevailing current is from south to north, and locations upstream from the sensitive zone could be influenced by the sensitive zone, but the extent is unknown. Locations at Klein Bonaire are regarded as "relative" reference as they lay further away from the coast of Bonaire.

Groundwater outflow can reach the reef at various depth, but where is unknown. Therefore it is advised to include two sampling depths per location: -20 m and -6m.

In Table II an overview is provided of the selected locations, and the assumed influences, including whether the location could be indicative for the effectiveness of the treatment plant. The locations are geographically ordered from north to south.

Table II Overview of selected locations

Location	**Outflow enriched groundwater**	**Other influence**	**Treatment plant**	**Reference location**
Playa Funchi	No	Indirect via wind/currents, Salinas	No	Yes (relative)
Karpata	No	Indirect via wind/currents	No	Yes (relative)
Habitat	Yes, with sewage	Yes (fertilisers, brine effluent WEB)	Yes	No
Playa Lechi	Yes, with sewage	Yes (yachts)	Yes	No
18th Palm	Yes, with sewage	Yes (yachts, fertilisers)	Yes	No
Angel City	Yes, but not from sewage	Yes, via Cargill salt pans	No	No
Cargill	Yes, but not from sewage	Yes, via Cargill salt pans	No	No
Red Slave	Yes, but not from sewage	No?	No	No
Ebo's Special (Klein Bonaire)	No	Indirect via wind/currents?	No	Yes
South Bay (Klein Bonaire)	No	No	No	Yes

As the preliminary treatment plant already started, and the extended treatment plant facility will be operational within several months it is advised to conduct the baseline monitoring as soon as possible in order to retrieve baseline data[1].

[1] Time of writing was September 2011

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1 Introduction

1.1 General introduction

On the island Bonaire, eutrophication is a point of serious concern, affecting the coral reefs in the marine park. Eutrophication can cause altered balance of the reef system because algae shall outcompete corals, eventually leading to a disturbed composition of the reef.

The reef of Bonaire faces nutrient input by various sources:

- Enriched groundwater outflow to the reef. Enrichment of groundwater is caused by:.
    - Sewage leaking from septic tanks.. Estimated is that a total of 118.275 m^3/year[2] flows into the reef ecosystem (Anonymous, 2008).
    - Fertilizers in resort gardens
- Run off via salinas and storm water
- Illegal discharge and overflows of septic tanks
- Discharge of yachts+ cruise ships
- Industrial discharge (e.g. salt company and WEB)

In order to reduce the input of nutrients via sewage water, a program was established to build a water treatment plant on Bonaire. Recently a preliminary treatment plant was built treating 200 m^3 a day (73000 m^3 a year). The treatment of sewage water will be extended next year (2012) with a sewage system covering the so called sensitive area, from Hato to Punt Vierkant (see Figure 1). This treatment plant, located at LVV near Lagun, is capable of treating 1200 m^3 a day (438000 m^3 a year). Van Kekem et al. 2006 estimated that the total nitrogen balance shows a total reduction of nitrogen input due to the foreseen treatment plant (with 2006 specifications) being 6.5 tonnes per year in the sensitive zone (by the year 2017 compared to 2005). Based on MIC, 2011 average influent conditions in practice are however assumed to be different (Table 1). Based on the details in table 1, it can be assumed that a total of 17520-35040 kg of Nitrogen is removed from the sensitive zone, and will not leach out to the sea at the western coast of Bonaire. The effluent will be discharged at the LVV area or used as irrigation water for agriculture. Part of the effluent might discharge to the sea at the eastern coastline, or infiltrates into the groundwater. Fate is then unknown.

Table 1 Assumed influent and effluent conditions (MIC, 2011)

Aspect	**Specification**	**Equals to**
Average flow rate	480 m3/day	175200 m3/year
Influent Total Nitrogen	100-200 mg/l	17520-35040 kg/year
Influent total Phosphorus	75-200 mg/l	13140-35040 kg/year
Effluent Total Nitrogen	46 mg/l	
Effluent total Phosphorus	65 mg/l	

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[2] This equals roughly to 21 m3/hour (in case of constant flow, which is not the case due to variable outflow).

Figure 1 *Map of Bonaire. Stars indicate the boundaries of the sensitive zone between Hato (north) and Punt vierkant (south)*

At the moment limited information is available about the total amount of nutrients in the marine environment, and the amount of nutrients per source. Van Kekem et al (2006) performed a mass balance study for Bonaire on Nitrogen. The seawater data are however based on a single sampling event at three locations. Additional field studies have been performed by Lapointe & Mallin in 2008, but the results have not been reported yet.

Rijkswaterstaat Waterdienst asked IMARES to conduct a study to:

1. suggest a program to monitor eutrophication in the marine environment of Bonaire in which the relation to the treatment plant can be made clear ;
2. Conduct a baseline study based on this program;
3. based on the results, advise on a monitoring program for upcoming years

The work is conducted in corporation with local organisations as DROB and STINAPA.

1.2 This report

The aim of this report is to define recommendations for monitoring the, expected positive, impact of the new sewage treatment system on the marine environment of Bonaire, with special emphasis on baseline monitoring. For this an evaluation was made of

- Parameters to analyse, argumentation, critical conditions
- Methods for sampling and critical conditions, including costs
- Potential sampling locations

2 Methods

The selection of indicators and sampling locations was done using several criteria.
Criteria have been applied to the overall suit of possibilities, in order to steer the discussion on final selection.
Criteria for indicators:

- Indicative for water quality cq eutrophication
- Indicative for effectiveness treatment plant
- Indicative for specific sources
- Background values and natural variance
- Applicability in terms of logistics in field and laboratory
- Quality assurance
- Costs

Criteria for selection of sampling locations:

- Geographical difference
- Influence of sewage sources (via groundwater)
- Influence of other factors
- Current
- Data availability previous studies

The selection of indicators is based on literature review, on previous research (e.g. by Lapointe & Mallin, in prep. and Slijkerman et al., 2011), expert knowledge obtained via various (local) experts (DROB, STINAPA, IMARES, Waterdienst, NIOZ, NIOO). Methods for sampling and analysis of the indicators was obtained via experience in the area, and evaluated by interviewing analytical laboratories with specific knowledge of type of sampling and critical conditions.

Selection of sampling locations is based on previous research and discussions with local expects (Ramon de Leon).

3 Results: Indicators

3.1 Indicator selection

Indicators related to sewage treatment in relation to environmental water quality have been selected. Expert knowledge, previous research by Lapointe & Mallin (in prep), Wieggers (2007), Slijkerman et al (2011) and local expert discussions (workshop October 2011 with Frank van Slobbe, Ramon de Leon, Kris Kats) resulted in the following indicators to be further evaluated in this section:

- General water quality parameters
- Nutrient concentrations (N, P)
- Chlorophyll a concentrations
- Stable isotope δ15N
- Benthic composition
- Fecal bacteria numbers

Each indicator is evaluated on the following aspects:

- Indicative for water quality cq. eutrophication
- Indicative for effectiveness treatment plant
- Indicative for specific other sources
- Background values and natural variance, including standards
- Need and critical conditions (e.g. equipment, methods, skills, preservation of samples and transport conditions)
- Quality assurance
- Costs

Information on needs, critical conditions, quality assurance and costs are included in annex 1.

3.2 General water quality parameters

3.2.1 Indicative for water quality cq eutrophication

Dissolved oxygen, pH, temperature, salinity, and turbidity are general water parameters to include in monitoring in order to assess general aspects (Table 2).

Table 2 Overview of water quality parameters

Indicator	Relation to water quality and environmental health
Dissolved oxygen	Oxygen concentration is related to water discharge and eutrophication. A low concentration is indicative for a low water quality. Quality standard for Bonaire: DO > 5 mg/L (Werkgroep Milieunormering Nederlandse Antillen, 2007)
pH	Increased primary production will lead to a shift in the CO2-carbonate equilibrium resulting in an increasing pH. However, more aspects influence pH (e.g. ocean acidiphication)
Temperature	General
Salinity (measured through conductivity)	Large freshwater discharge might result locally in lower salinity
Turbidity	Higher turbidity can be an indication for elevated algal growth (as result of nutrient input) and/or run off of sediments

3.2.2 Indicative for effectiveness treatment plant

These parameters are supporting general water quality indicators, and do not solely reflect the influence of the treatment plant. These parameters should be regarded in the context of other parameters.

3.2.3 Background values and natural variance, including standards

Water conditions around Bonaire are stable, with a constant 34-36 ppt salinity and mean annual water temperatures ranging from 26°C to 28°C (De Meyer, 1998). Regarding temperature, no standards have been provided in Milieunormen Bonaire (2007). However, temperature in tropical ecosystems is a critical parameter to monitor as it directly relates to coral bleaching. Bleaching occurs when the average sea surface temperature (SST) is 1°C above average seasonal maxima. Temperatures of 29°C are thus assumed critical. Although no local measures to avoid high temperatures can be taken, monitoring of temperature will help to interpret other observations.

For oxygen an environmental quality standard is provided by Milieunormen Bonaire (2007). Dissolved oxygen should be above 5 mg/L, and a stand still principles applies to those situations where water quality is above the standard. Background values for dissolved oxygen are around 8-9 mg/L (pers comm. Deleon).

Studies on background variation and standards for pH and turbidity in Bonaire or surrounding waters are not found in literature. An indication of background value of pH is ~8 (pers comm. Deleon, and Bonnélye et al., 2007).
According to (Hernández-Delgado et al., 2005) a chronic turbidity of 3 NTU could be detrimental to corals. Seasonal and temporal variation of turbidity is likely to occur due to rain events and run off from land. Turbidity values of 0.2-- 5.5 NTU have been reported in coral reef systems.

3.2.4 Indicative for other sources

These parameters are integrative indicators and are influence by various aspects in the marine environment, both ecological as human induced.

3.3 Nutrient concentrations in coastal zone

3.3.1 Indicative for water quality cq eutrophication

Nutrient poor waters are a requirement for healthy coral reefs. When these become enriched with nutrients, it results e.g. in increased algae and affected reef condition.
Parameters for the suit of nutrients are:

- N-NO2
- N-NO3
- N-NH4
- TIN Total Inorganic Nitrogen (sum NO2, NO3, NH4)
- Kjeldal –N
- P-PO4
- Total Phosphorus

Nitrite is usually regarded as an indicator of sewage pollution and is an intermediate product of the exudation process of ammonia (nitrification) and the reduction of nitrate (denitrification). Factors controlling nitrite are dissolved oxygen, microbial activity and the quantity and quality of wastewater.

Nitrate is the final oxidation product of nitrogen compounds, and considered to be controlling primary production.

Ammonia is one of the four forms of nitrogen compounds present in wastewater. Ammonia is an important source of nitrogen and may be assimilated to nitrate. In the marine environment ammonia is released as the degradation product. Under oxygenated conditions ammonia is rapidly oxidized by bacteria to nitrite which is in turn even more rapidly oxidized to nitrate.
Kjeldahl-N is indicative for sewage total N. The TKN-value(Total Kjeldahl Nitrogen) provides the sum of total nitrogen in the form of organic N en NH4 (TKN = org.N + NH4-N [mg/L]). In wastewater nitrogen is mostly only available in this form. After treatment, TKN is transformed into nitrite.
Phosphorus is one of the most important nutrients and is present in very small amounts in the local seawater. Therefor it plays a major role in phytoplankton growth as it limits productivity. In higher concentrations it may cause eutrophication.

3.3.2 Indicative for effectiveness treatment plant

Volumes of nutrients discharged are expected to decrease as result of the sewage water treatment. Based on Van Kekem et al (2006), a maximum reduction of 30% of nitrogen can be expected. PO4 is less indicative as PO4 absorbs to the soil and will not reach the marine environment unless the absorption capacity is maximised.

3.3.3 Background values and natural variance, including standards

Nutrient concentrations in tropical areas are very low due to oligotrophic conditions and constant fluxes in primary production cycle. Temporal variation is expected over the seasons. During dry season, biologically available nitrogen is 3-9 times higher than the maximum recommended for coral reefs, while during wet season values are 2-6.4 times the maximum. Biologically available phosphorous is also high, 1-8 times the maximum during dry season, 2-13 times during wet season (Gavio et al., 2010). Diurnal variability in nutrient concentration could be a factor as well (Gast et al., 1999).

Values measured in the coastal zone of Bonaire by Van Kekem et al., 2006 are noted in Table 3. In figure 2, values of phosphate and nitrogen are presented, based on an intern research project at CIEE, Bonaire. Values were obtained at 6 locations varying in sewage load (assumption). No significant variation between locations was found.

*Table 3 Average (n=3) nitrate and phosphate concentrations in seawater in mg/L. Standard deviation in brackets. Data by Van Kekem et al., 2006. * total P in west coast is lower than P-PO4. This indicates inaccuracy in sampling or processing of samples.*

	NO3-N	**NH4-N**	**Total N**	**PO4-P**	**P total**
West coast	0.3 (0.5)	0.06 (0.02)	0.6 (0.6)	0.06 (0.03)	<0.02*
East coast	<0.03	<0.04	<0.3	<0.02	<0.02

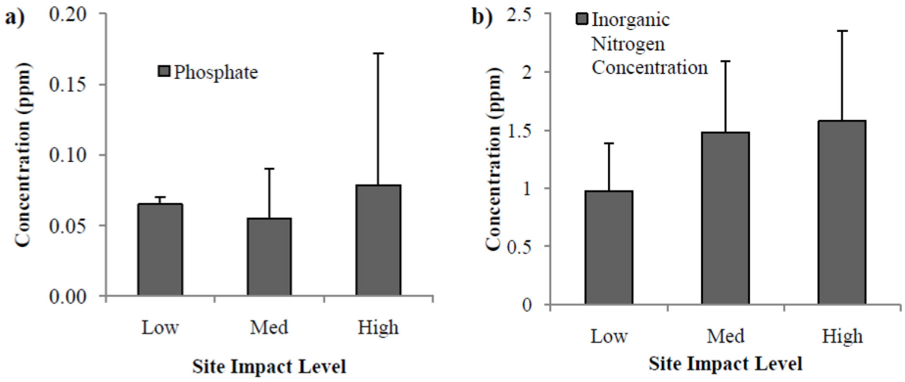

Figure 2 Background values of phosphate and nitrogen (DIN) by Giampetro, intern at CIEE (2010).

Wieggers (2007) found average values in seawater for NH4 of 0.77 µM N/L (= 0.0108 mg/L). The highest were found at the coastal zone near Salt Company, followed by Red Slave, Angel City and Ebo's Special. For PO4, Wieggers (2007) reported that most values were closely around 0.10 µM P/L (= 0.0031 mg/L). The levels of Angel City and Ebo's Special are above 0.15 µM P/L (=0.0046 mg/L). Total dissolved phosphor (TDP) was on average 0.26 µM/L (= 0.0081 mg/L). TDP levels showed highest values for the Salt Company. Average value DIN was 1.31 µM N/L. NO2 + NO3 values were on average 0.54 µM/L (= 0.0076 mg N/L).

Values by Lapointe & Malin (in prep) were not yet available for this study.

Thresholds for open coral reef systems have been established since the early 1990s (Bell, 1991, 2005, Lapointe et al., 1993). These thresholds are equivalent to the Standards Bonaire (Werkgroep Milieunormering Nederlandse Antillen, 2007) of 0.014 mg/L N (1.0 µM NH_4 or NO_2 + NO_3) or 0.003 mg/L P (0.1 µM P-PO4). Furthermore, the "stand still " principle applies to nutrient levels in the waters of Bonaire (Werkgroep Milieunormering Nederlandse Antillen, 2007). This means that if concentrations are above the standards, no detoriation of the water quality is accepted.

Quality standards Bonaire:

1.0 micromoles per litre of nitrogen as nitrate and ammonia
0.1 micromoles per litre of phosphorous as ortho-phosphate.

These values are in the molecular concentration units used by chemists and oceanographers. In the weight units more often used in the wastewater literature these values are equal to:

Nitrogen: 0.014 mg/L N or 0.040 mg/L NO3
Phosphorous 0.003 mg/L P

(mg/L= ppm)

3.3.4 Indicative for other sources

Groundwater is an important source of nutrients to coral reefs, especially where septic tanks are in use (Lapointe, 1997).

Run off of storm water on Bonaire is probably a significant source of nutrients via salinas and canals. The extent and amount of nutrients is not known. Other sources are fertilizers in resort gardens that enters the groundwater and via outflow reaches the reef, illegal discharge and overflows of septic tanks, discharge of yachts and cruise ships, and industrial discharge (e.g. salt company and WEB). The magnitude of these sources in unknown.

3.4 Chlorophyll a

3.4.1 Indicative for water quality cq. eutrophication

Elevated nutrient levels steer primary production and thus algal growth. This is reflected by increased phytoplankton chlorophyll in the water column, and/or increased biomass of benthic (macro-)algae. Phytoplankton chlorophyll-a has been used as an estimate of trophic status in aquatic systems and is a good indicator of nutrient loading by nitrogen and phosphorus. A better understanding of the mechanisms and trends of phytoplankton successions or ‘‘blooms’’ and their interactions with human activities may play an important role in the monitoring of coastal eutrophication (Linton and Warner, 2003). Chlorophyll a appears to be one of the best water quality indicators of eutrophication (Bell, 1992).

3.4.2 Indicative for treatment plant

Treatment of sewage and predicted decrease of nutrient levels in the coastal zone of Bonaire should result in lower chlorophyll a levels.

3.4.3 Indicative for other sources

Chlorophyll a levels are a net result of algal growth and grazing upon. Growth of alga is steered by light and nutrients. Increase of one or both factors results in elevated algal growth. Grazing by plankton or (jelly-)fish controls algal growth. Changes in chlorophyll *a* can be a result of increased nutrient concentration due to e.g. run off of sediments and organic material, or increased atmospheric deposition. Run off on Bonaire is probably a significant source of nutrients via salinas and canals. The extent and amount of nutrients is not known.

3.4.4 Background values and natural variance, including standards

Lapointe (in prep) and Wieggers (2007) report values of average 0.19 µg chlorophyll-a /L at various locations on the west coast of Bonaire in their study of 2007. No clear variation among locations was observed (Wieggers, 2007).
An environmental standard for chlorophyll a is not set by Milieudienst (Werkgroep Milieunormering Nederlandse Antillen, 2007). Bell (1992) suggests an eutrophication threshold value at or below an annual mean of 0.5 µg/L for these ecosystems, based on international research on coral reefs.

3.5 Stable Isotopes

3.5.1 Indicative for water quality cq eutrophication

An objective, cost-effective, assessment methods of sewage stress is the measurement of stable nitrogen isotope ratios, $\delta 15N$, in tissues of reef organisms (e.g. Risk et al., 2009, Lapointe et al., 2004). The choice of target organism will depend upon study purpose, availability, and other considerations such as conservation. Algae are usually plentiful and have been shown faithfully to track sewage input.

Wastewater nutrients from animal wastes, septic systems, and sewage treatment plants are generally enriched in the heavier isotope, 15N. This is due to nitrogen transformations that typically occur prior to or after discharge of such wastes. These transformations include ammonia volatilization, denitrification of nitrate, and nitrification of ammonia, all of which leave residual dissolved inorganic nitrogen (DIN) with high δ15N (after Risk et al., 2009, and: Heaton, 1986; Heikoop et al., 2000).
δ15N is found to be an excellent indicator of sewage input. Values of δ15N decrease away from sources, generally reaching background levels after about 10–15 km (Risk et al., 2009), of course depending on local circumstances.

The dilution-due to distance- principle can be used to study the effectiveness of the treatment plant as well, but instead of using distance as dilution factor, time is used. Over the years, a drop of total δ15N to the reef can be expected due to discharge of sewage to the treatment plant. This should result in lower δ15N in tissue of organisms. It is however yet unsure how much time it will take to detect this drop in δ15N in tissue (if detected).

Factors influencing the detection are:

- Natural variance among species,
- Variance within tissue of specimen
- Seasonal variance in run off of δ15N due to rain
- Increase of outflow due increased sewage (in total).

It is advised to analyse δ15N in macro algae tissue as algae are the first users of N and sessile macro algae give location specific information. Especially chlorophytes such as *Codium* sp., or phaeophytes such as *Dictyota sp. Sargassum, Lobophora, Cladophora, Codium*) are potential good species. An overview of species and corresponding δ15N under sewage stress is provided in annex 2.
Furthermore, benthic macro algae have been used in studies all over the world and have been shown to be particularly abundant, easy to sample in coral reef environments and have straightforward analysis (within Risk et al., 2009: Lapointe, 1997; Umezawa et al., 2002; Lapointe et al., 2004, 2005; Lin et al., 2007).
Macro algae are typically attached to the substrate and integrate nutrient availability over time scales of days to weeks. They can act as indicators, not only of nutrient quantity (tissue % C, N, and P), but also of nutrient source (tissue δ15N and δ 13C). This allows researchers to use macro algae to quantify and fingerprint land-based nitrogen inputs (Risk et al., 2009). The macro algae would provide spatial data (within one sample moment), or over time when sampled in time series. In Figure 3 data show the decrease of δ 15N in macro algae over distance.

There is a further, political/practical advantage to using macro algae: many of the world's reefs are being overgrown by macro algae, a process the origins of which are hotly debated. Macro algae may be sampled with the comforting knowledge that no international treaties are being breached, and no government agency or conservation NGO will be looking over the diver's shoulder (Risk et al., 2009).

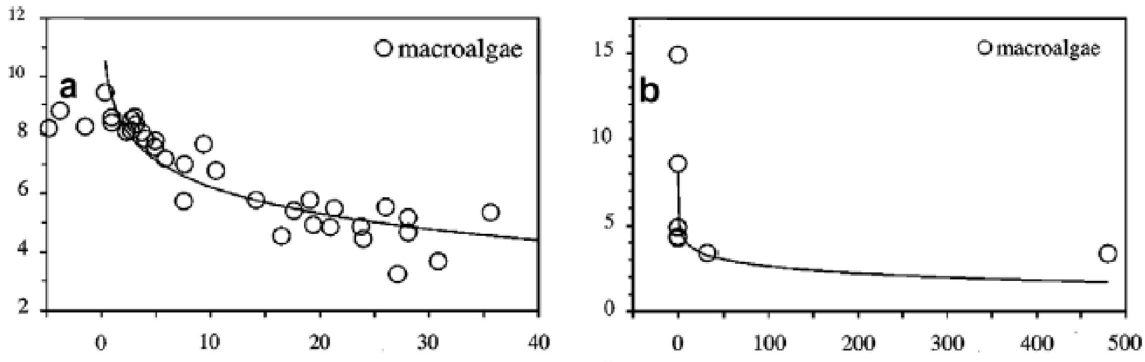

Figure 3 *Isotope δ15N (‰) in macro algae vs distance to source (x-axes) Figure from Risk et al 2009.*

An alternative tissue to consider are barnacles as they also show clear responses in isotope values to distance from outflow (Risk et al., 2009), see Figure 4.

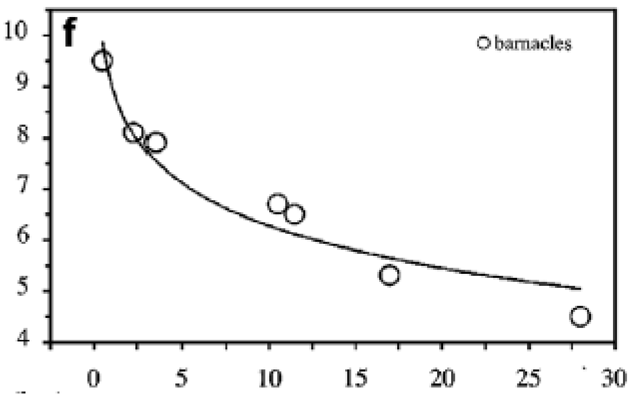

Figure 4 *Isotope δ15N (‰) in barnacles vs distance to source (km). Figure from Risk et al 2009.*

Drawbacks and limitations to take into account;

- Tissue turn-over rates can be high: "time integrated" fingerprinting maximum of months, probable lower, depending on the species.
- Scale of sampling of macro algae can be a difficult factor, particularly when other (natural) major N sources to the reefs in question are not included within the sampling design. As δ15N levels are strongly related to N source values, the major N source (s) for a given water mass must be known and characterized. This is especially important when comparing different water masses (e.g. shallow coastal waters vs. upwelled waters). Along developed coastlines with significant agricultural activity, the addition of inorganic fertilizer with low δ15N values will complicate any search for a sewage signal. As well upwelling or other N sources might interfere with sources of study.
- δ15N levels are potentially affected by microbial processes such as nitrification and denitrification. Additional data on nitrogen species, such as ammonium and nitrate concentrations, are useful for interpreting results, particularly for macro algae that have a high affinity for ammonium. NH4 and NO3 should thus be analysed as co-variance factor to be able to interpret data (Lapointe et al., 2005).

3.5.2 Indicative for treatment plant

See above section.

3.5.3 Background values and natural variance, including standards

The western coastal area of Bonaire stretches over approximately 35 km. The extent of sewage influence might completely cover this region, with highest δ15N in Kralendijk region, a dilution effect to the north. Lowest δ15N can be expected in the south due to the prevailing currents providing incoming unpolluted water at the southern tip. Any reduction in δ 15N as result from the treatment plant should be best visible at locations near shore in the sensitive zone.

Draft data of Lapointe & Mallin (in prep) retrieved in 2006-2008 can be used as a preliminary baseline for δ15N. However, data do not show clear baseline or strict distinguishing between locations yet (Table 4).

Table 4 Summary of δ15N (‰) in macro algae in data of Lapointe & Mallin (2006-2008)

date	**δ15N min (‰)**	**δ15N max (‰)**	**locations max value**	**location min value**	**Remarks**
Mar-06	0	12.4	Playa Lechi + Lagun	South bay + Playa Funchi	All location avg <1.4
Jun-06	0.3	2.2	Playa Funchi + Angel City	Ebo's special	All locations avg <1.6
Oct-06	0.3	3.7	Front Porch + Playa Lechi	Ebo's special + South bay	Most (-1) locations avg <1.9
Jan-07	0.2	5.1	Playa Lechi	South bay	All locations avg < 2.1
Mar-07	0.3	3.1	Ebo's special+ Front porch	18th Palm	All locations avg < 2.4
Jul-07	0.4	6.4	Lagun+ Red slave	South Bay + Ebo's special	Most (-1) locations avg <2
Feb-08	0	2.7	Red Slave + Karpata	Ebo's special + Playa Lechi	All locations avg < 1.7

In general, all locations show on average values below 2.4 ‰. In literature (see annex 2) these values can be regarded slightly impacted. Lagun, Playa Lechi, front porch and Red Slave show highest values, values which in literature are reported as impacted by sewage. Lagun was not included in all sampling dates. When Lagun is included, it shows high values. Ebo's Special and South Bay show lowest values and are clearly not impacted by sewage. However, this test was not significant, as variance was high within locations.
This variance might be due to different reasons:

- Sampling variance (number of samples, species, tissue)
- Seasonal variance within and among species
- Seasonal variance in run off quality and volume
- Depth as factor not yet taken into account. Seems not to be very discriminating among 15 and 60 ft samples. Shallow samples (1 feet) show highest δ15N values and seems like important discriminating sampling depth.

Optional carbon isotope ratios can be added in the analysis as C and N isotope ratios can be used to discriminate between marine and terrestrial based organic matter. C and N can therefore be used to detect terrestrially formed organic matter from sewage effluent in the marine environment (Rogers 2003).
The source of carbon isotope via groundwater outflow is however judged of less discriminating then δ15N.
Furthermore, preparation of samples for C isotope analysis is much more labour intensive due to the need for prevention of carbonate interaction. Samples need to be pre-conditioned.

3.5.4 Indicative for other sources

Sewage is a very clear source for increased isotope ratios. Increased δ15N can be linked to other sources as well such as fertilizers or animal feces. See annex 2 for an overview.

3.6 Fecal bacteria

3.6.1 Indicative for water quality cq eutrophication

Sources of fecal bacteria on Bonaire are diverse. Septic tanks might leak or overflow into the groundwater, illegal discharge of sewage, overflow during storm events, and bathing tourists. Enterococci are a proxi for monitoring fecal bacteria in marine waters because they are persistent in saltwater whereas. Coliforms such as *Escherichia coli* experience high rates of decay in seawater (Anderson et al., 1979). The occurrence of gastroenteritis among users of marine bathing waters has been directly related to levels of enterococci, which are the only indicator bacteria used to monitor water quality in the US (EPA, 1986). Enterococci are quantitatively linked to illnesses in swimmers (Kay et al. 1994, Wade et al., 2003) and levels are monitored in countries around the world to issue warnings when densities exceed threshold values.

3.6.2 Indicative to treatment plant

Volumes of polluted groundwater are expected to decrease as result of the installation of the water treatment plant. Bacteria levels are expected to decrease consequently.

3.6.3 Background values and natural variance, including standards

Coliform bacteria do not live long in seawater, therefore they are only indicative for recent pollution events.
In the study of Slijkerman et al (2011) in Lac, levels of enterococci at marine sites ranged from undetectable to 399 cfu/100 ml, with a mean for bay stations of 39.5 ± 0.04 cfu/100 ml on the incoming tide (n = 32) and a mean of 45.1 ± 90.1cfu/100 ml on the outgoing tide (n = 32).
For 2 well sites that are just inland of the bay (15 – 600 m), enterococci ranged from 3.1 to 658.6 cfu/100 ml, which is well within the range of enterococci detected in the well water of Bonaire, which in recent testing exceeded 2,400 cfu/100 ml (R. Peachey, unpublished data).

Water measurements taken during the field visit by Vermeij (2011) indicate a large number of pathogenic bacteria (Vibrio spp.; > 1600 bacteria per ml) to be present in the water (normal concentrations are 0-10 bacteria per ml). Heavy rains occurred in this period and might have caused overflows from septic tanks.

European bathing water standard is 185 cfu/100 ml for a minimal acceptance, and 100 cfu/100 ml for a good quality (based on resp. 95 percentile and 90 percentile over multiple samples).
US EPA standard (Criteria for Bathing (Full Body Contact) Recreational Waters) is based on a statistically sufficient number of samples (generally not less than 5 samples equally spaced over a 30-day period) for which the geometric mean of the Enterococci densities should not exceed 35 cfu/100 ml.
Caribbean blue flag criterium is <100 cfu/100 ml (fecal coliforms) in minimum 75% of samples taken over a period of a year and <400/100 ml in any sample.

3.6.4 Indicative for other sources

Enterococci might come from other sources then sewage and septic tanks. Stormwater including cattle and bird feces, as well as bathers themselves add to fecal bacteria. Sewage from yachts might be another source of bacteria input.

3.7 Benthic composition

This indicator is covered by annual monitoring executed by STINAPA and not discussed in detail in this report.

3.7.1 Indicative for water quality cq eutrophication

Benthic composition is the overall quality indicator to all human and natural influences. Eutrophication , toxicity and continues physical disturbance will lead to disturbed and changed benthic composition.

3.7.2 Indicative for effectiveness treatment plant

Specific parameters within the benthic composition survey, could be very indicative for increased nutrient input, e.g.:

- Percentage Coral cover
- Percentage Algal cover
- Presence of Coral diseases/mortality
- Density of Coral bio eroders, especially sponges
- Density of Macro invertebrates as sea urchins

These parameters should be assessed in combination with other steering factors (see 3.7.4) such as fish abundance (grazers, predators).

3.7.3 Background values and natural variance, including standards

Historical data and trend analysis are available, but limited. Meesters & Van Beek, (in prep) have reported draft functional maps of the west coast of Bonaire, and could be used. Also STINAPA conducted a benthic survey in 2011, but results are to be processed. Data of STINAPA and Meesters & Van Beek can both be used as baseline. Natural variance is not included in these data.

3.7.4 Indicative for other sources

Benthic composition is steered via different factors, both natural or human.

- Temperature rising (global change) leading to bleaching events
- Hurricanes
- Geological coverage
- Diseases
- Sedimentation
- Nutrient input
- Grazing/predation alteration of fish/benthos via e.g. over-fishing

3.8 Nutrient concentrations in groundwater, effluent and influent

Additional to environmental monitoring, the effectiveness of the treatment plant can be monitored via other direct analysis of:

- Nutrient concentrations in influent and effluent (before and after sewage treatment).
- Nutrient and bacteria concentrations in groundwater (sampling via boreholes/piezometers)

The quality of influent and effluent are the most direct quality parameters to include monitoring in order to assess the effectiveness of the treatment plant. The total reduction of N and P should be measured in samples taken over time in order to account for variable quality. When related to the total volumes of treated sewage the total reduction of N and P tot the reef can be calculated.

The groundwater will be directly influenced by the installation of the treatment plant and treated sewage which should result in reduced faecal bacteria numbers and nutrient concentrations . Via so called boreholes or piezometers, groundwater samples can be taken. These boreholes should be placed in the sensitive zone, and near reference areas. The difficulty of this set up is steered by soil characteristics of Bonaire. The groundwater outflow is heterogeneous due to the calcareous soil and placement of boreholes is critical. A pilot set up is advised, in which a screen of boreholes in included.
Parameters to include are nutrients (NH_4, NO_2, NO_3, PO_4, Total P and kjeldahl N) and bacteria numbers. Detection limits are less critical as expected concentrations are higher, and the matrix is brackish/freshwater. When sampling near the coast, salinity of the water samples can be higher. When contracting laboratories these variable salinity aspects should be taken into account and communicated to the contracting party.

This monitoring isn't part of the environmental monitoring of 2011, but should be regarded as valuable additional monitoring in future in order to identify causal relationships.

3.9 Transport of samples

Critical conditions and protocols for sampling and processing of samples are described in annex 2.
Transport of samples to the laboratory is the main critical condition in any environment, but even more under tropical conditions. Exposure to sunlight and heating of the samples should be avoided to the maximum. Directly after sampling of water, the samples should be kept on ice in a cooler. Processing time should be kept as short as possible, but at least within hours..
Once in the lab, the samples should be stored in the refrigerator. After processing, the samples for nutrients and chlorophyll should be stored kept in the freezer at -20°C. Samples should be transported at -20°C to the laboratory at which analysis takes place.
The transport from Bonaire to any laboratory includes the risk of defreezing the samples. A transport route should be as short as possible, with a minimum of transfer handling to minimise the risk of delay. Transport to US laboratories are assumed to be of higher risk than European laboratories due to custom restrictions. Personal communication with US lab's learned that this was a critical aspect at incoming samples at these lab's. Often the samples were defrozen due to delay at customs.

Transport with dry-ice is explored, but not yet feasible at the terms of the first monitoring. On Bonaire no dry ice is available. It could be retrieved from Curaçao but not yet operational. The permit system should be taken care off.

Transport of samples in hand or checked in luggage is a common manner amongst scientist to get samples from the Antilles to Europe. The direct flight to Amsterdam is a suitable manner of transporting samples at once. A suitable cool box and cool packs are needed to keep the samples frozen at all times.

Options for transport to the Netherlands are when not using checked in luggage: :

- Larry's transport services. Using direct KLM flights from Bonaire- Amsterdam. 9 hour flight, plus check in time.

4 Results: Locations

Location selection should be based on several criteria:

- Geographical difference, including currents
- Influence of sewage sources (via groundwater)
- Influence of other factors
- Data availability from previous studies
- * groundwater sampling station not yet included
- Reference location

<u>Geographical difference:</u>
Locations along the coast of Bonaire differ in composition and quality (northern vs southern reef system) and influence of sewage and this should be covered for in the monitoring.
Taking into account currents and eddies only general information is available. Bonaire is located close to where Atlantic water flushes into the Caribbean Basin through the leeward island chain. Bonaire lies down stream of surface water flow from the direction of St. Vincent and the Grenadines and wind driven currents from Las Roques and Las Aves. When the surface currents strike Bonaire on the windward shore, they are deflected to the north and south. There are pronounced eddies at the south of the island (near Willemstoren), at the north of the island (Malmok and Boca Bartol) and just north of BOPEC. Currents are unpredictable but slight, rarely exceeding 0.5 m s-1. The predominant current movement is toward the north along the leeward shore, but this pattern is complicated by local eddies and upwelling (see
Figure 5). Water conditions are stable, with a constant 34-36 ppt salinity and mean annual water temperatures ranging from 26°C to 28°C (De Meyer, 1998).

Figure 5 Map of Bonaire with prevailing current and known locations of eddies. The red circle roughly indicates the 'sensitive zone' being influenced by sewage.

Influence sewage sources

The influence of wastewater concentrates on the westcoast of Bonaire. A so-called "sensitive zone" is delineated extending 200 m inland from the coast, between Punt Vierkant and Hato (

Figure 5). In this area, wastewater is produced in large quantities. The majority of this wastewater ends up in the marine environment via outflow, run off, irrigation or percolation. Within the sensitive area, at least 118275 m3/year of wastewater from hotels reaches the marine environment. In this calculation residential properties and small businesses are not included, making the amount an underestimation. Locations within the sensitive zone should be included as being "worst case" locations. At these locations all indicators are expected to show the strongest response to the installation of the treatment plant.

Data availability:

Locations of relevant previous research are e.g. Lapointe & Mallin (in prep), Wieggers 2007, Slijkerman et al. (2011) and Meesters & Van Beek (in prep). Slijkerman et al focused on Lac bay. Meesters & Van Beek (in prep) covered the whole west coast on a 500 m grid, including Klein Bonaire resulting in a functional map of Bonaire.

Reference locations

Locations outside the sensitive zone can be regarded as reference. Locations outside sensitive zone and downstream of the prevailing currents are even more pristine/reference then locations upstream, but can be influenced by the salt company. Locations at Klein Bonaire can be regarded as reference as they lay further away from the coast of Bonaire and the sensitive zone. However, draft results from Lapointe on isotope ratios show higher values at Ebo's special at one sampling moment. Influence from the sensitive zone cannot be completely excluded. In Table 5 an overview is provided of locations per study and their relation to the enriched area. In Figure 6 a map with the sampling location is shown.

*Table 5 Overview of locations and influence of nutrients and connection to the treatment plant. Locations are ordered from North to South. If locations are not indicated as influenced as such, they can be regarded as reference. This assumption is however a relative comparison. * LVV is the treatment plant area at which effluent is discharged*

Location	**Study**	**Outflow enriched groundwater**	**Other influence**	**Sensitive zone**	**Treatment plant area**	**Reference**
Playa Funchi	Lapointe, Wieggers	No	Indirect via wind/currents	No	No	Yes
Karpata	Lapointe	No	Indirect via wind/currents	No	No	Yes
Habitat	Lapointe, Wieggers	Yes, from sewage	Y (fertilisers)	Yes	Yes	No
Playa Lechi	Lapointe, Wieggers	Yes, from sewage	Yes (Yachts)	Yes	Yes	No
Front Porch	Lapointe, Wieggers	Yes, from sewage	Yes, yachts	Yes	Yes	No
18th Palm	Lapointe, Wieggers	Yes, from sewage	Yes (yachts, fertilisers)	Yes	Yes	No
Red Slave	Lapointe, Wieggers	Yes, from salt pans	No	No	No	No
Angel City	Lapointe, Wieggers	Yes, from salt pans	No	No	No	No
Cargill	Wieggers	Yes, from salt pans	No	No	No	No
Cargill channel	Wieggers	?	Yes, via salt	No	No	No
Cargill pond	Wieggers	?	Yes, via salt	No	No	No
Ebo's Special (klein Bonaire)	Lapointe, Wieggers	No	Indirect via wind/currents	No	No	Yes
South Bay (klein Bonaire)	Lapointe, Wieggers	No	No	No	No	Yes
Lagun (eastcoast) *	Lapointe	Yes	No	No	Yes, via LVV*	No
Lac	Slijkerman et al., 2011	?	Yes (via sewage and manure)	No	No	Other

Eastcoast is influenced via Lagoon area. Monitoring is not feasible on this coast by scuba and shore entrance due to rough conditions.

Wieggers (2007) reported two sampled depth at each location. Depending on the variable, depth can be a discriminating factor. It is thus advised to include two depths per location , 20 m (60 ft) and a shallow location (starting of the reef, being -6m).

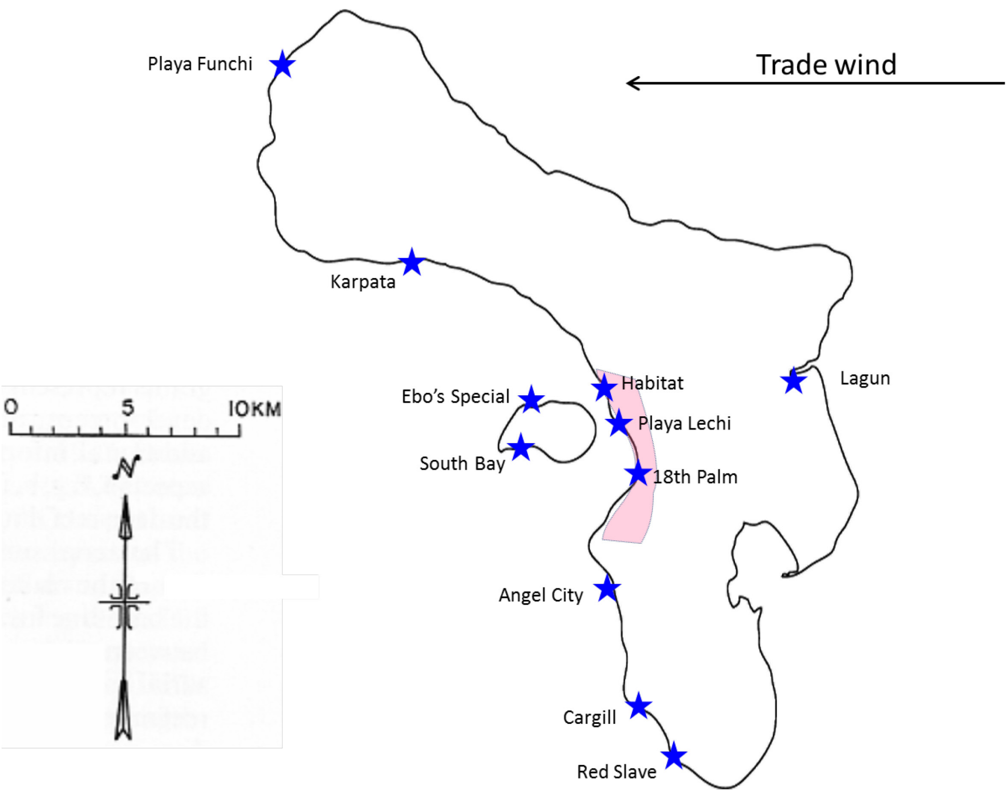

Figure 6 Map of Bonaire with the selected sampling locations.

5 Recommendations

5.1 Indicators and laboratories

Based on their relevance to the treatment plant it is advice is to explore the following parameters in the first (pilot) monitoring:

- Inorganic nutrients (thus not Kjehldahl and total P)
    - NO_2, NO_3, NH_4, PO_4.
    - DIN is calculated based on NO_2+ NO_3+ NH_4
- General water parameters, including dissolved oxygen, pH, temperature and turbidity
- Stable isotope $\delta^{15}N$ in macro algae
- Chlorophyll a
- Fecal Bacteria (using Enterolert test kit)

Based on logistics, critical conditions and limitations of risks during transport, it is advised to contract laboratories at Bonaire and in the Netherlands. On site laboratory to prepare samples should cover for practical lab conditions which is space, access to distilled or Milli Q- water, general supplies as stove, sealer, incubator, refrigerator and freezer.

All prepared samples (nutrients, macro algae and chlorophyll a samples) can be transported in once on a direct flight to the Netherlands by checked in luggage. The following laboratories are advised to contract:

- Bonaire CIEE: On site laboratory to prepare samples, general laboratory supplies including a multimeter
- NIOZ: analysis of stable isotopes
- NIOO: analysis of nutrients
- IMARES: analysis of chlorophyll a

5.2 Locations

Advised is to include 11 locations, mostly based on historical study of Lapointe and Wieggers. These sites cover all geographical differences (northern and southern reef, Klein Bonaire), and include locations inside and outside the sensitive zone. Locations outside the sensitive zone can be regarded as a (relative) reference. Locations outside the sensitive zone and downstream of the prevailing currents are can be influenced by the salt company.
Front Porch is not included as Habitat, 18th Palm and Playa Lechi are locations with similar environmental conditions, and all lay within the sensitive zone. Three out of four locations that look alike is considered sufficient. Cargill/salt company as a location is included based on high nutrient level measured by Wieggers (2007).
Lagun is added as 11th location at which only surface water sampling will be conducted. Lagun is a location influenced directly by effluent and sewage by the treatment plant. Sampling by means of scuba is not done at this location.

1. Playa Funchi
2. Karpata
3. Habitat
4. Playa Lechi
5. 18th palm
6. Angel city
7. Red slave
8. Cargill/ salt company

9. Ebo's special
10. South bay
11. * Lagun (only surface water due to risk of diving)

Groundwater outflow can be expected at various depths. Sampling is advised to conduct at two sampling depths (-20 m and -6 m) to account for variable water quality in the shallow and deeper reef zone.

5.3 Timing

As the preliminary treatment plant already started, and the extended treatment plant facility will be operational within several months it is advised to conduct the baseline monitoring as soon as possible in order to retrieve best baseline data[3].

[3] Time of writing was September 2011

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7 Quality Assurance

IMARES utilises an ISO 9001:2008 certified quality management system (certificate number: 57846-2009-AQ-NLD-RvA). This certificate is valid until 15 December 2012. The organisation has been certified since 27 February 2001. The certification was issued by DNV Certification B.V. Furthermore, the chemical laboratory of the Environmental Division has NEN-AND-ISO/IEC 17025:2005 accreditation for test laboratories with number L097. This accreditation is valid until 27 March 2013 and was first issued on 27 March 1997. Accreditation was granted by the Council for Accreditation.

8 Justification

Report C027/12
Project Number: 430.51096.01

The scientific quality of this report has been peer reviewed by a colleague scientist and the head of the department of IMARES.

Approved: E.M. Foekema
Researcher

Signature:

Date: March 15th 2012

Approved: F.C. Groenendijk, MSc.
Head of Department

Signature:

Date: March 15th 2012

Annex 1 Needs and critical conditions, Quality assurance

A.1-1 Water parameters

Needs and critical conditions

In Table 6 an overview is provided of needed material.

Table 6 overview of needed material

Needed	Total	Critical condition	availability	remarks
Multimeter, with oxygen, pH, temperature probe	1	To be calibrated	STINAPA, PROES, CIEE	To be used only with instructed personnel
Turbidity meter (fluorometer or multimeter)	1	To be calibrated	CIEE	To be used only with instructed personnel

Quality assurance

Calibration of multimeter and proper use according to user manual before each new measurement (regarded when meter has been switched off).

A.1-2 Fecal Bacteria

Needs and critical conditions

Analysis via NEN-norms is not possible as water samples cannot be preserved and has to be processed within 24 hours. This analysis technique is not available on Bonaire or nearby. The Enterolert system (IDEXX, Philadelphia PA) provides a good alternative.

Needed:
- Clean lab space
- Sterile sample bottles 100 ml
- Sterile pipettes
- enterolert kit Quanti-Tray®/2000
- Quanti-trays
- sealer
- Incubator 41°C
- UV lamp
- MPN chart
- Ethanol
- Sterile water

<u>In the field</u>
Samples are collected in sterile bottles of which 10 ml is pipetted (sterile) into a 100 ml polyethylene bottle and stored on ice pending transport to the laboratory.

In the lab
The 10 ml sample of the seawater is filled to the 100 ml with sterile freshwater, and Enterolert reagent is added. Gently shake the sample until reagent is dissolved. Samples are poured into a Quanti-tray, sealed and stored in an incubator at 41°C for 24 h. Quanti-tray cells were examined under a 365 nm UV light (blacklight) for fluorescence and interpreted according to the mean probable number table supplied by IDEXX.

Quality assurance

Enterolert is U.S. EPA-approved and is included in Standard Methods for Examination of Water and Wastewater.Enterolert is an official ASTM Method (#D6503-99).
Sensitive to 1 enterococci/100 mL
Enumerates up to 2,419 enterococci per 100 mL without dilutions (with Quanti-Tray®/2000)
Less subjective interpretation
50% fewer false positives and 95% fewer false negatives than the standard membrane filtration (MF) method
Under one minute hands-on time
Results in 24 hours

A.1-3 Stable isotopes

Needs and critical conditions

Based on Lapointe et al. (2004) and personal communication with Lapointe and NIOZ laboratory personnel a protocol has been established. In Table 7 an overview of needed supplies is given.

Table 7 overview of needed supplies for sample processing prior to isotope analysis.

needed	**total**	**remarks**
Plastic zipbags	Multiple ~90	
Mortar and pestle (porcelain)	1	
Dry oven 60 °C	1	
Balance 1 µg accurate	1	Present at CIEE, not at IMARES
Tin cups	Multiple ~90	Size depends on sample volume
96 Well tray	1 or 2	Depends on total samples
Lab Spates		
Tweezers		
Cover tape		
refridgerator		

In the field
At each location at two depths sampling of two key (most abundant) species; Try as much for same algal species, e,g *Dyctiota, Halimeda, Lobophora*. Collect minimum of 5 individuals per species.
Store in marked plastic bag and place in coolbox during transport to the laboratory.

In the lab
Cut same type of tissue of each individual macroalgae. E.g stem, root, leave. Be consequent in selection of tissue type over all samplings among locations and among sampling dates.

Number of samples:
Per location a maximum of 2 species per depth is collected resulting in 4 samples per location. Each sample is homogenised, and 2 subsamples taken out of each sample.

Acceptable range of sample weights is based on the results the 2006-2008 study of Lapointe (1%en 2% N in samples).
For ^{15}N analysis based on 1% N:

- Smallest sample weight (mg): 2.00
- Optimal sample weight (mg): 10.00
- Largest sample weight (mg): 15.00

Based on 2%:

- 1 vs 5 vs 7,5 mg

Quality assurance

Different laboratories are identified which can analyse stable isotopes in plant tissue. Different laboratories were contacted, but not all responded.

- NIOZ
- Stable isotope lab Davis (US)
- RUG: did not respond in time to take into account

NIOZ laboratory was chosen based on responsiveness, costs and logistics. At NIOZ, the nitrogen isotopic composition of organic matter was determined using a Thermofinnigan Delta V isotope ratio mass spectrometer connected on-line to a Carlo Erba Instruments Flash 1112 elemental analyzer. All isotope abundances are given in conventional delta notation in the per mill scales versus air N2. The nitrogen isotopic composition was calibrated against laboratory standard acetanilide (δ15N= 1.3%) and checked against laboratory standard L-Glutamine (δ15N=-4.5). Reproducibility of the isotopic analysis was determined by multiple analysis of the lab standards and found to be better than 0.25 per mill.

A.1-4 Nutrients

Needs and critical conditions

In Table 8 an overview is provided of material needs, and specifications prior to use.

Table 8 Overview of material needs for nutrient analysis.

needed	**Total**	**Specifics**	**To be discussed**	**Present**
Aquet	1	Needed for rinsing new bottles		IMARES
HCl	10%	Needed for rinsing between sampling		CIEE
Sample bottles of 500 ml-1L:	minimum of 12, based on 2 locations each day, 2 depths per location and triplicate sampling. (2*2*3)	Rinsed prior to use.		IMARES

needed	Total	Specifics	To be discussed	Present
Sub sample plastic bottles of 20-100 ml (depend on lab) + screw caps	Minimum of 60	Rinsed prior to use		IMARES
Syringes for 60 ml	Minimum of 60	Rinsed prior to use	Use multiple times = risk of pollution	IMARES
Filters 0.2 µm	Minimum of 60	Rinsed prior to use.	Based on Gast et al (1998)	IMARES
Cool box	Volume for 12 litre bottles, and cool packs	-		STINAPA or CIEE
Coolpacks	Multiple for in box	-		STINAPA or CIEE
Refrigerator	1	To store the samples prior to handling		CIEE
Freezer	1	To preserve samples for 4 weeks **at -20C**		CIEE

Because of the low ambient concentrations, contamination is a point of attention during sampling and handling of the samples. Sampling should be conducted with care.
A triplicate sampling is advised during the baseline study to account for sampling variance. Evaluation of data should discuss if triplicate sampling can be reduced to duplicate sampling.

<u>In the field</u>
Sampling: Rinse each bottle and cap 3 times with location water prior to definite sampling.
Be aware NOT to touch inside of the cap or bottle.
Take total of **3 bottles of water (triplicate)** at each location on two depths 20 ft (ridge of reef) and 60 ft. Sampling at both depths just above seafloor (cm).

Store litre bottles in coolbox directly after taking the samples and store in lab refrigerator once there. Record the collection data on the Sample Collection Form. Note anything that could influence sample chemistry (heavy rain, potential contaminants) in the Comments section. If the samples were not taken at the X-site, enter the GPS coordinates of the sampling location and the reason for relocation in the comments field on the Sample Collection Form

<u>In the laboratory</u>
Before subsampling: Resuspense 1 litre bottle by turning over
Take 1 subsample from each sample bottle.
Use individual syringe and filter for each sample, and do not touch the filter and syringe tip with hands.
Fill the syringe halve, shake and discard water.
Repeat 3 times
Fill syringe again fully and screw filter on
Flush sample through the filter. This cleans the filter.
Fill syringe and fill sub-sample jar of 20 ml, and rinse jar 3 times before definite subsampling.
Mark the sample with unique code
Freeze the sample to -20°C for a maximum of 6 months prior to analysis, but preferably analyse within 4 weeks.

After handling and preserving rinse each sampling bottle with Aquet and tapwater and rinse 3 times with 10% HCl, following MilliQ.

Quality assurance and laboratory overview

To report relevant nutrient levels, and too be able to evaluate these levels to current standards, detection (quantitation) limits should be lower than the environmental standards. Standards are given in total N and total P. Individual N is advised to be reported as different forms of nitrogen are needed to evaluate ecological impact. In Table 9 an overview is provided of laboratories consulted. Methods, quantitation limits, costs , risks and quality assurance is taken into account.

NIOZ laboratory is best choice based on quantitation limits and quality control. However, NIOZ laboratory are renovating their buildings and do not accept third party samples. Therefore, NIOO laboratory is advised based on the provided quantitation limits, low volume needed, and least risk of defreezing due to one way transport route (BON-AMS).
The low prescribed volume is beneficial during preparation of the samples as filtering of water takes much time.
CIEE lab could be considered for longer term analysis if quality assurance is provided.

Table 9 Laboratory overview. Methods, suitability of detection limits, costs, quality assurance and risks. Laboratories on Caribbean islands (Barbados, St Maarten, Curacao, and Bonaire) have been explored but not found suitable in this respect.

Lab	Method	ml sample needed	NH4	NO2	NO3	PO4	Costs	Quality assurance	Risks
NIOO	QuAAtro continuous flow analyser	6	yes	yes	yes	yes	60 EUR	ring test	Defreezing during transport
NIOZ	See NIOO	2	yes	yes	yes	yes	No contracting	ring test	-
WL	-		yes	no	no	yes		NEN	No proper detection limit
WD	-	250	yes	no	no	yes		NEN	No proper detection limit
Omegam	-	250	yes	no	no	yes		NEN	
CIEE Bonaire	Turner fluorometer	10	Yes, to be tested	no	Yes, to be tested	Yes, to be tested	Not yet indicated	Not yet, could be ring test or internal standard	No standardisation yet
Maryland	Bertholet Reaction, Cd reduction, conform EPA	100	Yes 0.01mg/L NH4-N	Yes 0.0035 mg/L NO2+ NO3-N		Yes 0.0025 mg/L PO4-P	40 USD	Ring test	-Defreeze during transport via multiple routes -US-customs

A.1-5 Chlorophyll a

Needs and critical conditions

- 12 1 litre bottles, dark colored.
- Filters
- Alu foil
- Cool samples directly after sampling.

Quality assurance

In the field
See nutrient sampling, but in dark bottles of 1 litre.

In the laboratory
Filter for each sample a total of 500 ml with the syringe used for nutrient filtering.
If 500 ml does not succeed due to clogging of the filter, note down the total of ml filtered. This is needed to calculate the amount of chlorophyll per litre.
Fold and store the filter in alu-foil and freeze at -20 °C until analysis for a maximum of 6 months (Aminot and Rey, 2000)

Analysis of Chlorophyll a at IMARES laboratory is performed by means of aceton extraction. Detection limit can be as good as 0.1μg/L, but depends on total chlorophyll filtered (personal comm. Pascalle Jakobs).

A.1-6 Costs

Costs for the analysis of mentioned indicators depend on various factors. Total expenses –without costs for analysis and reporting– are estimated for T0 on EUR 13030,-. This is based on previous experiences and prices obtained at 3rd parties. The following costs are included:

- Sampling material: ~1000 EUR
- Sampling and processing time on site/Lab: depends on total days. To be set on 9000 EUR and to be evaluated in report 2.
- Transport costs: ~500 EUR (depends on weight and taxes)
- Fee CIEE lab (depends on total days and number of guest personnel): 520 EUR
- Lodging and travel 1 person: ~2000 EUR
- Benthic composition is already performed by STINAPA on yearly bases. No additional costs are yet foreseen.
- Multimeters are available through STINAPA, Proes or CIEE and otherwise through IMARES. No costs for purchasing a multimeter are foreseen.
- Laboratory, analysis of indicator by third party: see Table 10 and Table 11

Analysis of indicators by IMARES and third parties costs 498 EUR per location (Table 11). Based on the advice to sample at 11 locations a total budget of 5478 EUR is needed to account for the analysis.

The estimated costs for expenses, sampling, processing and analysis for T0 are: 18508 EUR.

Additional expenses for Enterolert-test if CIEE laboratory could not be used:

- 1 x Quanti Tray Sealer 3.050 Euro
- 1 x UV lamp 120 Euro
- 1x incubator 4000 EUR

Table 10 Costs per indicator per sample for various suppliers. Only analysis costs are shown, no costs for sampling and other expenses. In yellow the costs for the supplier chosen.

Type analysis	lab/suplier	per sample		In EUR	remarks	
Nutrients	Woodshole	18	USD	€ 13.33	Suit of NH4, NO2 Po4. no T-p and NO3	Costums to US is high risk
Nutrients	Maryland	38.5	USD	€ 28.52	NO2, NO3, NH4, Po4 and total P	Costums to US is high risk
Nutrients	Waterdienst	internal costs			NH4 en PO4, kjeldahl en total P	
Nutrients	**NIOO**	**60**		**€ 60.00**	NH4, NO2, NO3, PO4	
Stable Isotopes 15N	Stable isotope lab	7	USD	€ 5.19		Costums to US is high risk
Stable Isotopes 15N	**NIOZ**	**15**	**EURO**	**€ 15.00**		
Chlorophyll a	Maryland	12.5	USD	€ 9.26		Costums to US is high risk
Chlorophyll a	Miami	8	USD	€ 5.93		Costums to US is high risk
Chlorophyll a	**IMARES**	**7**	**EUR**	**€ 7.00**		
Chlorophyll a	WD	internal costs				
Bacteria via enterolert	**Enterolert**	**6**	**EUR**	**€ 6.00**		

Table 11 Costs per location, based on costs for laboratory analysis. No costs for travel, and laboratory time included.

indicator	depths	replicates	samples per location	costs per location
nutrients	2	3	6	€ 360.00
chlorophyll	2	3	6	€ 42.00
isotopes*	2	2	4	€ 60.00
bacteria	2	3	6	€ 36.00
				€ 498.00
* estimate, depends on actual sampling				

Annex 2 Overview of δ15 N ‰ values

Source	Research description	Location	Species	15N ‰	Circumstances	Notes
Lapointe et al (2004)	Land-based nutrient pollution as a significant human threat to coral reefs.	Shallow seagrass and coral reef communities between the Content Keys (southern Florida Bay) and Looe Key (south of Big Pine Key) in the Lower Florida Keys	*L. intricata*	4.69 ‰, ± 1.14	inshore area directly impacted by sewage discharges	elevated NH4 + concentrations in the water column were associated with elevated d15N values in macroalgae at levels reported for nitrogenous pollution from sewage and/or agricultural runoff.
"	"	"	*L. intricata*	3.03 ‰, ± 0.46	nearshore patch reef located inshore of Hawk Channel	
"	"	"	*L. intricata*	3.00 ‰, ± 1.04	offshore bank reef at Looe Key	
"	"	"	*Cladophora catenata*	3-5 ‰	an offshore bank reef at Looe Key. the d15N values of C. catenata increased from < + 3.0 per mille to > + 5.00 per mille following the onset of heavy rain and increases in DIN; the d15N values increased again from values < + 1.00 per mille to > + 5.00 per mille following high northeast winds and DIN enrichment	
"	"	"	*T. testudinum*	4.20 ‰, ± 1.10 in the dry season and + 3.62 ‰, ± 0.32 in the wet season		
"	"		*T. testudinum*	3.45 ‰, ± 1.04 in the dry season and + 3.80 ‰, ± 0.69 in the wet season		
"	"		*T. testudinum*	2.88 ‰, ± 0.74 ‰ in the dry season and 3.26 ‰ the wet season		

Source	Research description	Location	Species	15N ‰	Circumstances	Notes
"	"	"	macroalgae	> 3 ‰, sewage nitrogen	Wet season, three inshore sites Bird Island, Pine channel, Content Keys.	
"	"	"	macroalgae	< 2 ‰	Wet season, LK back reef and fore reef stations	
"	"	"	macroalgae	overall mean of 2.46 ‰, ± 0.97	entire transect from Content Keys to LK	
"	"	"	macroalgae	5.93 ‰, ± 0.59	Dry season, all stations	
Heaton, 1986	This paper reviews the mannes in which studies of natural abundance 15N/14N ratios may be employed in investigating the sources and mechanisms of pollution.	-		1.0 -3.0 ‰	Agricultural fertilizers and peat nitrogen	values characteristic of nitrogen derived from agricultural fertilizers and peat nitrogen
"	"	-			High 15N values > 10 per mille = localised pollution by animal or sewage waste	
Lapointe et al (2004)		Shallow seagrass and coral reef communities between the Content Keys (southern Florida Bay) and Looe Key (south of Big Pine Key) in the Lower Florida Keys	macroalgae and seagrass epiphytes	3.0 - 5.0 ‰	chronic sewage inputs at AJ	values characteristic of nitrogen derived from septic tanks and/or cesspits runoff
Lapointe and Thacker, 2002		Negril Marine Park, Jamaica	macroalgal communities (*Sargassum, Lobophora, Cladophora, Codium*)	4 to 6.5 ‰	shallow reefs adjacent to urbanized areas and wastewater discharges	lowest values consistently occurred at the most offshore station least impacted by landbased, elevated NH4 + concentrations on shallow reefs were significantly higher than on deep reefs, suggesting sewage and agricultural runoff as land based sources of nitrogen pollution to the Negril Marine Park

Source	Research description	Location	Species	15N ‰	Circumstances	Notes
Lapointe and Thacker, 2002		Negril Marine Park, Jamaica	macroalgal communities (*Sargassum, Lobophora, Cladophora, Codium*)	0.5 to 2.0‰	deep reefs less exposed to wastewater discharges	
Lapointe et al., 2010		Tobago, West Indies	macroalgae	11 to 12 ‰	on fringing reefs at a sewage outfall in Buccoo Bay	
"		Tobago, West Indies	macroalgae	6.0 ‰	Buccoo Reef Complex	
"		Tobago, West Indies	macroalgae	3.0 ‰	most offshore site off Little Tobage island	
Barile and Lapointe, 2005		Abacos, Bahamas	macroalgae	8.0 ‰	sewage polluted harbor	
		Abacos, Bahamas	macroalgae	2.0 ‰	offshore reefs	
Mendes et al. 1997		Kingston Harbor, Jamaica	*M. annularis*	6.4 ‰	on reefs most impacted by sewage	
"		Kingston Harbor, Jamaica	*M. annularis*	1.5 ‰	offshore reference site	
Lapointe, 1997		eutrophic coral reefs off southeast Florida	*Codium isthmocladum*		increased from ~ + 5x to + 11x following heavy summer rainfall and increased discharges of sewage-contaminated groundwaters enriched in 15N into the Loxahatchee River and coastal waters	
France et al., 1998		coastal waters of southwestern Puerto Rico	21 samples of tropical macroalgae	4.0. to 6.0 ‰	relatively unpolluted coastal waters	a value close to the atmospheric signature of 0x and indicative of nitrogen fixation as the source of nitrogen supporting growth
McClelland et al., 1997		Waquoit Bay watershed estuary system, Cape Cod, Massachusetts		2–8 ‰	groundwater nitrate influenced only by atmospheric deposition	

Source	Research description	Location	Species	15N ‰	Circumstances	Notes
Costanzo et al., 2001	A technique that identifies the source, extent and fate of biologically available sewage nitrogen in coastal marine ecosystems. This method is based on the uptake of sewage nitrogen by marine plants and subsequent analysis of the sewage signature (elevated 15N) in plant tissues.	near sewage outfalls in Moreton Bay, Australia, a semi-closed bay receiving multiple sewage inputs.	a seagrass *Zostera capricorni*, attached macroalgae *Gracilaria edulis, Catenella nipae*, mangrove *Avicennia marina*.	10 ‰	15N signature of treated sewage	
McClelland et al., 1997		Waquoit Bay watershed estuary system, Cape Cod, Massachusetts		10–20 ‰	Groundwater nitrate generated from human and animal wastes shows heavier values	
McClelland et al., 1997		Waquoit Bay watershed estuary system, Cape Cod, Massachusetts		- 3 ‰ to 3 ‰	Synthetic fertilizers	
Kuramoto and Minagawa, 2000		Southwestern Coast of Thailand		8 ‰	Previous work has also reported that particulate organic nitrogen (PON) from estuarine waters surrounded by densely populated areas tends to have a δ15N higher than 8‰ because organic waste from human residential areas often enhances denitrification in drainage systems.	
Heaton, 1986		-		>8‰	High nitrogen loads into river-systems are often associated with elevated δ^{15}N values in dissolved inorganic nitrogen (DIN), particulate organic matter (POM) and macroalgae	
Wassenaar, 1995 and Kendall, 1998.		Wassenaar, 1995: the Fraser Lowlands both in southwestern British Columbia (B.C.), Canada, and northern Washington State, U.S.A.		> 10‰	Nitrate and ammonium originating from human and animal waste usually show δ15N values 10 ‰ because of fractionation processes during transformation from one N-species to another.	

Source	Research description	Location	Species	15N ‰	Circumstances	Notes
McClelland and Valiela, 1998	To explore this link between anthropogenic N and primairy producers, stable isotope ratios of N in groundwater and producers from the Waquoit Bay watershed estuary system, Cape Cod, Massachusetts, were compared.	Waquoit Bay watershed estuary system, Cape Cod, Massachusetts		< 8 ‰	Rivers with low anthropogenic N load usually show δ15N values in DIN, POM and macroalgae < 8‰ which reflects nitrate and ammonium sources from atmospheric deposition or nitrate from nitrification in pristine soils.	
Kendall, 1998		-		-20 and +30‰.	terrestrial materials	
Kendall, 1998		-		0 ‰	The dominant source of nitrogen in most forested ecosystems is the atmosphere	
Kendall, 1998		-		0 ± 3‰ +10 to +25‰	Watersheds including fertilizers produced from atmospheric nitrogen animal manure	
Aravena et al., 1993		Yasu River and Ado river in the Lake Biwa Basin, Japan		- 2 to + 4 ‰ , +3 to +8 +10 to +20 for.	- commercial fertilizers -soil organic nitrogen nitrate - human and animal waste nitrate	
				5‰ to 9‰	The δ^{15}N of untreated wastewater	
Kreitler and Browning, 1983				10‰ and 22‰	treated wastewater	