Start-up of the system and monitoring requirements and specifications, test-plan

Deliverable 3.2

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Note: The submission of this delivery has been delayed for some months given that the timetable of the testing and optimization of the BIODAPH technology at the two demo-sites is included in it and in order to make it most realistic we waited until the construction and installation of the reactors finished in November 2023.

DISCLAIMER

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Acronyms

Acronym	Title
GA	Grant agreement
UdG	University of Girona
NTUA	National Technical University of Athens
CSIC	Consejo Superior de Investigaciones Científicas
HRT	Hydraulic retention time
WP	Work package
WWTP	Wastewater treatment plant
MCU	Monitoring control unit
COD	Chemical oxygen demand
TSS	Total suspended solids
BOD5	Biological oxygen demand (5 days)
ARGs	Antibiotic resistance genes
PFAs	Perfluoroalkyl and polyfluoroalkyl substances
EPA	Environmental protection agency

Executive Summary

This report is written within the framework of WP3– (Task 3.1.) of the BIODAPH$_2$O project under Grant Agreement No. 101074191. The participants in the project are the University of Girona (project coordinator), ACSA-Sorigué, the CSIC Research Institute, MINAVRA Techniki, the National Technical University of Athens (NTUA), the BETA Technological Centre and the Catalan Water Partnership (CWP).

LIFE BIODAPH$_2$O is a demonstration project which has the main objective of scaling-up and implementing an eco-efficient nature-based tertiary wastewater treatment (BIODAPH) at two demo sites located in water-stressed regions of the Mediterranean area (Catalonia and Greece). This system will produce reclaimed water that will contribute to diminish discharges of pollutants in freshwater ecosystems and promote agricultural reuse. The BIODAPH system, previously developed in the INNOQUA project (GA 689817), is based on the depuration capacity of biological organisms, water fleas (Daphnia), microalgae and biofilms, to remove pollutants. This compact and low-energy consumption system does not produce sludge nor uses chemicals in its operation.

Under WP3, this deliverable aims to describe the guidelines to be followed during the set-up and operation of the BIODAPH wastewater treatment system and the monitoring requirements to assess its efficiency. The deliverable also contains information on the test-plan for the optimization of the operational conditions to achieve the objectives of the BIODAPH$_2$O project.

Disclaimer

1 Introduction

The BIODAPH technology is based on the filtration capacity of Daphnia and other aquatic organisms such as *Chydoridae* and *Ostracoda*, as well as on the depuration capacity of microbial/algae biofilm. The biofilm will develop on both the side walls and constructed lamellas that are detailed in the final reactor designs in Deliverable 3.1, and shown in Figures 1 and 2, for the reactors in Quart (Spain) and Antissa (Greece), respectively.

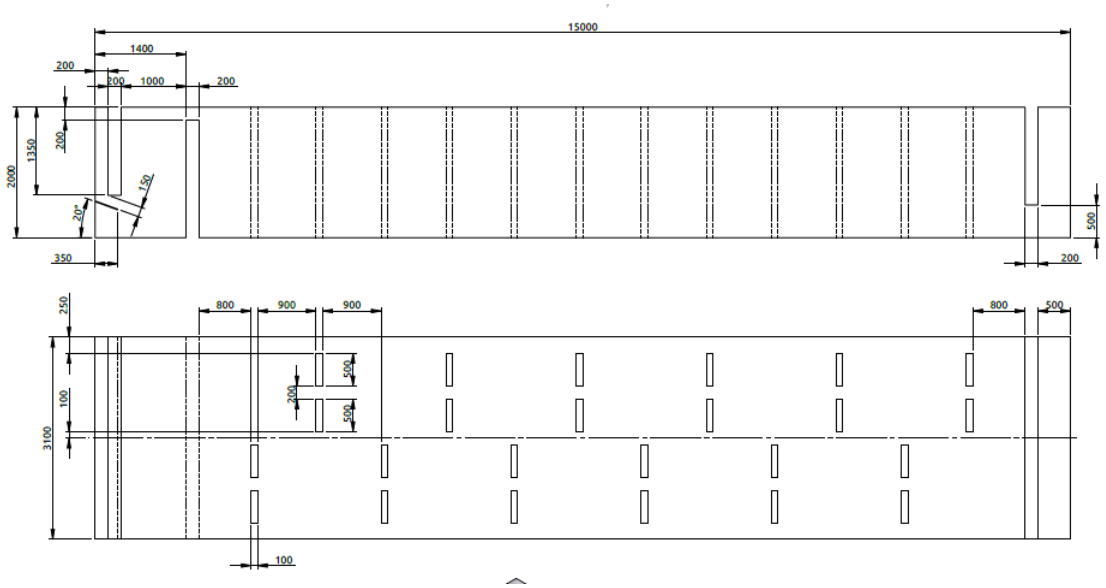

Figure 1 Side view (top) and top view (bottom) of the BIODAPH tank constructed in Quart (Spain). The tank is divided into three sections: The receiving waters section, the main body of the tank occupied by 12 lamellas, and the outlet tank.

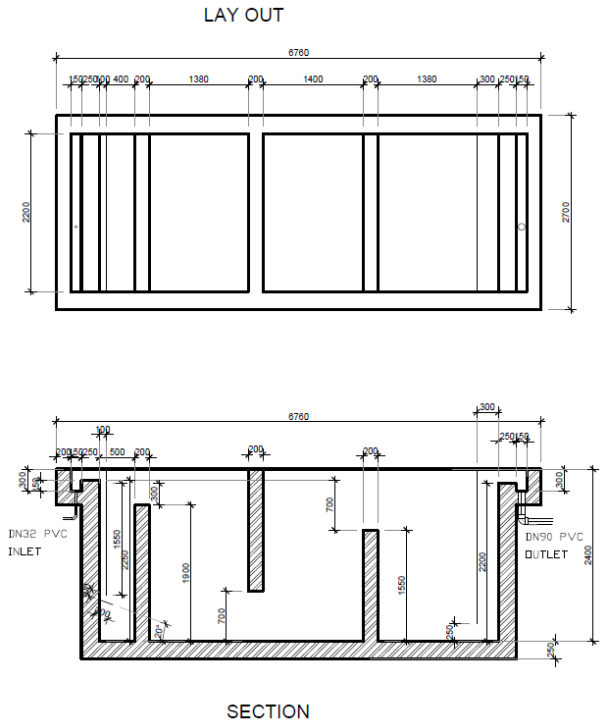

Figure 2 Side view (top) and top view (bottom) of the BIODAPH tank constructed in Antissa (Greece). The tank is divided into three sections: The receiving waters section, the main body of the tank occupied by 2 lamellas, and the outlet tank.

During tasks 3.3 and 3.4, the tanks will be filled with secondary wastewater in the Quart demo-site, and with constructed wetlands in the Antissa demo-site. In contact with secondary wastewater, microbacterial/algae biofilm will grow on the walls and lamellas of the BIODAPH reactors and Daphnia individuals will be introduced into the tanks when the environmental and chemical conditions inside the reactors will be favorable for the development of the Daphnia population. The individuals needed for inoculation of the reactors will be collected from *Daphnia magna* culture in a side inoculation tank, already operating at both WWTPs (Figure 3). The system will be left to evolve to steady conditions. The time lag is estimated to be in the order of 1-2 months and is based on previous studies made within the framework of the INNOQUA project (Pous et al., 2020, Pous et al., 2021, Salvadó et al., 2021, Serra et al., 2022). During this process, the main parameters, such as water temperature, dissolved oxygen, turbidity and pH, will be monitored.

Figure 3 Photograph of *Daphnia magna* individuals (red) captured from the inoculation tank (bottom) in the Quart WWTP in May 2023. Authors: Jordi Colomer and Aina Amengual.

1.1 Objective

The main objective is to optimize the operational conditions such as the hydraulic retention time and pollutant loading rates in order to enhance the purification capacity of the technology. The achievement of the most efficient conditions for operation will be evaluated in terms of the quality parameters required for the specific uses of the reclaimed water at the two demo-sites. In the case of Quart (Spain) the aim is to reduce the impact of secondary wastewater discharges into the river Onyar by improving the chemical and ecological quality of the aquatic ecosystems within the river allowing the standards set in Water Framework Directive of the EC (Directive 2000/60/EC) to be reached and reducing the discharge of emerging pollutants. In the case of Antissa (Greece) the aim is to reclaim water in accordance with EU Regulation 2020/741 to irrigate 7000 m^2 of nearby agricultural land.

2 Inoculation of *Daphnia magna* individuals and their maintenance

2.1 General Description

Water fleas (daphnids) are small crustaceans of the Cladocera order, normally found in lakes, rivers and other suitable freshwater habitats around the world. *Daphnia magna* is a common species of daphnids that is widely used for ecotoxicological studies (Bownik et al., 2017). They have been found to be able to remove small suspended particles (diameter < 30 µm) that have very low settling velocities. Furthermore, they have also been reported to reduce bacterial loads such as *E. coli* and coliforms (Serra et al. 2022). Given this, *Daphnia* are used in BIODAPH as a clarifier (reducing the suspended solid content) and disinfectant (reducing the bacterial load). The filtration capacity of *Daphnia* makes them suitable for use as a tertiary wastewater treatment (Pous et al. 2020, 2021a, 2021b). *Daphnia* could also contribute to reducing organic matter content, nutrients, and emerging contaminants through the consumption of particulate organic matter.

The ideal operating conditions are between 18ºC and 23ºC (water temperature). The minimum and maximum recommended operating conditions are between 10 ºC and 25 ºC (water temperature) (Müller et al. 2018; Schalau et al., 2008). Water temperatures out of this range will produce a decrease in the concentration of *Daphnia* and therefore the efficiency of the treatment will

decrease. In preliminary inhibition tests, *Daphnia* were found to be sensitive to nitrite and ammonium concentrations as seen in Figure 4 (Serra et al., 2019; Maceida-Veiga et al., 2015). The effect of the *Daphnia* exposure to different COD concentrations was evaluated by counting the *Daphnia* population after 24 h of exposition. *Daphnia* were also severely affected by COD, especially at concentrations of about 250 mg COD·L^{-1}. It implies that a good COD removal performance in the secondary treatment unit must be assured before transfer the secondary treated effluent to the BIODAPH reactor.

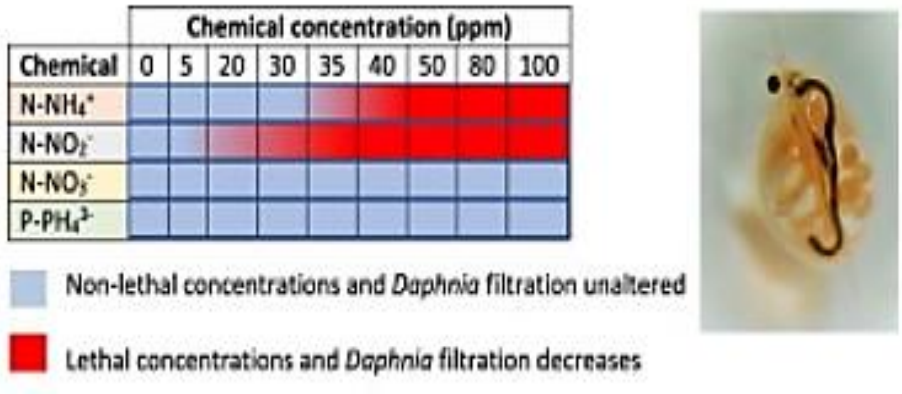

Figure 4 Effects of chemicals on the development of *Daphnia* individuals. On the right, a photograph of a *Daphnia magna* individual. Note that the gut is full of food and eggs are present (Serra et al. 2019a).

2.2 Inoculation and maintenance of the required conditions for Daphnia development.

Daphnia individuals will be introduced into the BIODAPH reactors at a concentration of 0.01 Daphnia.L^{-1}, which will give a total number of 1000 individuals in the Quart reactor, and 250 individuals in the Antissa reactor. The inoculation will be done after assessing the environmental and chemical conditions inside the reactors by measuring the main physical and chemical parameters (i.e temperature, pH, dissolved oxygen, TSS, COD and ammonium. Daphnia population will be left to evolve for a minimum of two weeks. The number of Daphnia individuals will be assessed following the procedure described in section 4,

The self-regulation of the Daphnia population inside the BIODAPH reactors is due to parthenogenesis, which is a natural form of asexual reproduction. The offspring are exact genetic replicas of the parent and any differences in the physical state of these clones is due to environmental conditions.

Parthenogenesis has evolved to allow *Daphnia* to take advantage of favourable conditions (food, temperature, etc) as soon as they arise. In the environment, during the late spring, summer and early autumn (depending on temperature, food availability and the presence of waste products of their metabolism), *Daphnia* reproduce by parthenogenesis, bearing, on average, ten live young individuals each (the entire population is made up of females during this period). Developing embryos are often visible in the mother's body without the aid of a microscope (brown colour eggs inside their body, see Figure 1). Successive generations of females can be born in this way, with new females reproducing as early as four days after occlusion at intervals as often as every three days, for up to twenty-five times in their lifetime (though this number is usually far smaller, and females tend to produce a lot fewer than one hundred offspring).

When food is scarce or *Daphnia* are under adverse environmental conditions, some black eggs develop into males and the females produce eggs that must be fertilised (the sexes reproduce via haploid means). These eggs develop into small embryos which then go into suspension and are shed with the carapace as dark brown/black saddle-shaped shells known as ephippia. These can survive harsh conditions and are quite capable of withstanding a dry spell and can sometimes even survive freezing. When conditions improve again, ephippia hatch and a new *Daphnia* is born, potentially leading to a new bloom of *Daphnia* if the environmental conditions are favourable. There are usually two of these pulses or blooms every year (one in spring and another in autumn), although there can be many more.

The life span of *Daphnia*, from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the species and environmental conditions. The time required to reach maturity varies from 6 to 10 days. Generally, the lifespan increases as temperature decreases, due to lowered metabolic activity. The average lifespan of *D. magna* is about 40 days at 25 °C and about 56 days at 20 °C. The average lifespan of *Daphnia pulex* at 20°C is approximately 50 days.

Worsening in the environmental and chemical conditions inside the reactors can affect the performance of Daphnia. Hence, the growth of filamentous algae must be prevented and removed if present and aerobic conditions must be guaranteed, especially in the case that feeding is interrupted due to mechanical failure. Despite of BIODAPH technology does not produce sludge, it is possible that some sludge will be accumulated at the bottom of the first chamber of the reactor in case of malfunctioning of the system, making necessary to clean it.

2.3 Shut-down Prevention Requirements for *Daphnia* culture

Under aerobic conditions, *Daphnia* can tolerate periods of up to two weeks without feeding. Relatively short shutdowns are not problematic for the system,

which can in many cases be restarted again without any specific intervention. In the case of long shut-down periods, the settled sludge, if any, must be removed to avoid high oxygen consumption due to excess organic matter in the system.

2.4 Maximizing the yield

There are several conditions that can affect the yield of *Daphnia* population:

Salinity - *Daphnia* are typically freshwater organisms and there are no marine species of the *Daphnia* genus. 99% of Cladocerans are found in freshwater, and the remaining few species are mostly found in brackish, rather than sea water (Schuytema et al., 1997). Some species have been observed in salinities up to 4 ppt, and salinities of 1.5 to 3.0 ppt are common in some pond cultures.

Oxygen - *Daphnia* are generally tolerant to poor water quality, and dissolved oxygen varies from almost zero to supersaturation (Lee et al., 2022). They are able to survive in oxygen-poor environments due to their capacity to synthesize haemoglobin, whose production may be promoted by high temperatures and high population density. However, when an aerator is used in the tank care must be taken to avoid over-agitating the water since calm conditions are required for the Daphnia to grow.

pH and ammonia - A pH between 6.5 and 9.5 is acceptable, with the optimum being between 7.2 and 8.5. Ammonia is generally highly toxic to all organisms, even in small amounts, but in alkaline conditions, the toxicity radically increases, and this will drastically impair *Daphnia* reproduction, although it will not affect the actual health of the animals themselves.

Daphnia are often used in toxicity tests as they are extremely sensitive to metal ions such as copper and zinc, and other dissolved toxins such as pesticides, detergents, bleaches as well as by high concentrations of microplastics (Magester et al. 2021). Therefore, *Daphnia* cannot be used as a primary or secondary treatment system, but instead have good performance when working as a tertiary treatment.

2.5 Daphnia culture in the laboratory

Daphnia can also be cultivated in the laboratory and this culture requires very little maintenance. The procedure we follow for the culture of Daphnia in the laboratory are set out in Annex I

3 Initial operation of the BIODAPH systems in Spain and Greece

The initial operation of the BIODAPH reactors will be at an HRT of 2 days. The BIODAPH systems will be primed to reach steady state over 2 weeks. The main purpose is for *Daphnia magna* individuals to adapt to the environmental parameters, including the chemical, physical and hydrodynamic conditions inside the reactor, and to allow the growing of the biofilm.

A MCU system will permanently monitor the following parameters: water temperature, pH, turbidity, and dissolved oxygen inside the Quart reactor. The main physicochemical parameters will be measured at the influent and effluent of the reactors.

The operation of the BIODAPH system in Spain consists of the tasks set out in Table 1.

Table 1 Description of Actions and periods for the Start-up in Spain and Greece

Time (month)	Action
November 23- M16	Start-up: Introduction of treated wastewater in the reactor for the growth of biofilm and development of optimal ecosystems (HRT=2)
December 23 -M17	Monitoring of water temperature, pH, dissolved oxygen, and turbidity to check if the conditions are good for Introducing Daphnia magna individuals for further development of optimal population (HRT=2).
January 24- M18	Monitoring of water temperature, pH, dissolved oxygen, turbidity.
January 24- M18	Counting of *Daphnia magna* individuals

4 Testing and optimization of the BIODAPH system

4.1 Testing and optimization of the BIODAPH system in Spain

After the initial operation (start-up) of the BIODAPH reactors, the next phase involves the testing and optimization of the system. The testing consists of six trials in which three HRTs, 1, 1.5 and 2 days, will be tested in order to optimize the operational conditions. The optimization criteria will be based on the compliance with the Spanish Reuse Royal Decree 1620/2007 and measurements will be carried out every 15 days.

First, the monitoring control will include the measurement of standard wastewater parameters such as COD, BOD5, TSS, ammonium nitrogen (NH_4-N), phosphate (PO_4-P), nitrate, and nitrite, in addition to water temperature, pH, dissolved oxygen, turbidity (which will be continuously monitored by the MCU).

Second, the monitoring control will include the measurement of microbiological quality (*Escherichia coli* and coliforms) parameters in compliance with the measurements will be carried out once every month.

The system needs a two-week period of stabilization after each HRT trial. Once equilibrium conditions are obtained and *Daphnia magna* population will be fully developed. Within this period, the maximum length of the individuals will be reached, maximizing the filtration of particles. For each trial, after stabilization, monitoring will be performed for two months.

The test plant will then include 48 samples that will be collected from December 2023 (M17) to November 2025 (M40).

In the case of emerging pollutants and pathogens, 3 sampling campaigns will be performed to analyse influent and effluent samples with five replicates per campaign, giving 30 samples. These campaigns are expected to be performed at the 3 HRTs during the optimization of the operational hydrodynamic conditions. Four groups of emerging contaminants: 1) pharmaceuticals, 2) ARGs, 3) microplastics, and 4) PFAs will be determined. Pharmaceuticals include diclofenac, ibuprofen, triclosan, sulfamethoxazole, trimethoprim, tetracycline, ciprofloxacin, and erythromycin. ARGs include sul1, tetW, ermB, qnrS, and blaTEM. The analysis of microplastics will include visual classification and counting, as well as the quantification of twelve polymers. Finally, 40 PFAs (EPA Draft Method 1633) will be determined.

Reactor biodiversity in crustacean and microalgae will be monitored by means of an optical microscope and using an appropriate classification atlas. The

phytoplankton biomass will be monitored by analysing the chlorophyll-a concentration in water samples.

The biological status of the biofilm inside the reactor will be measured on the lamellas each month. The status of the biofilm includes photosynthetic efficiency and phototrophic community composition.

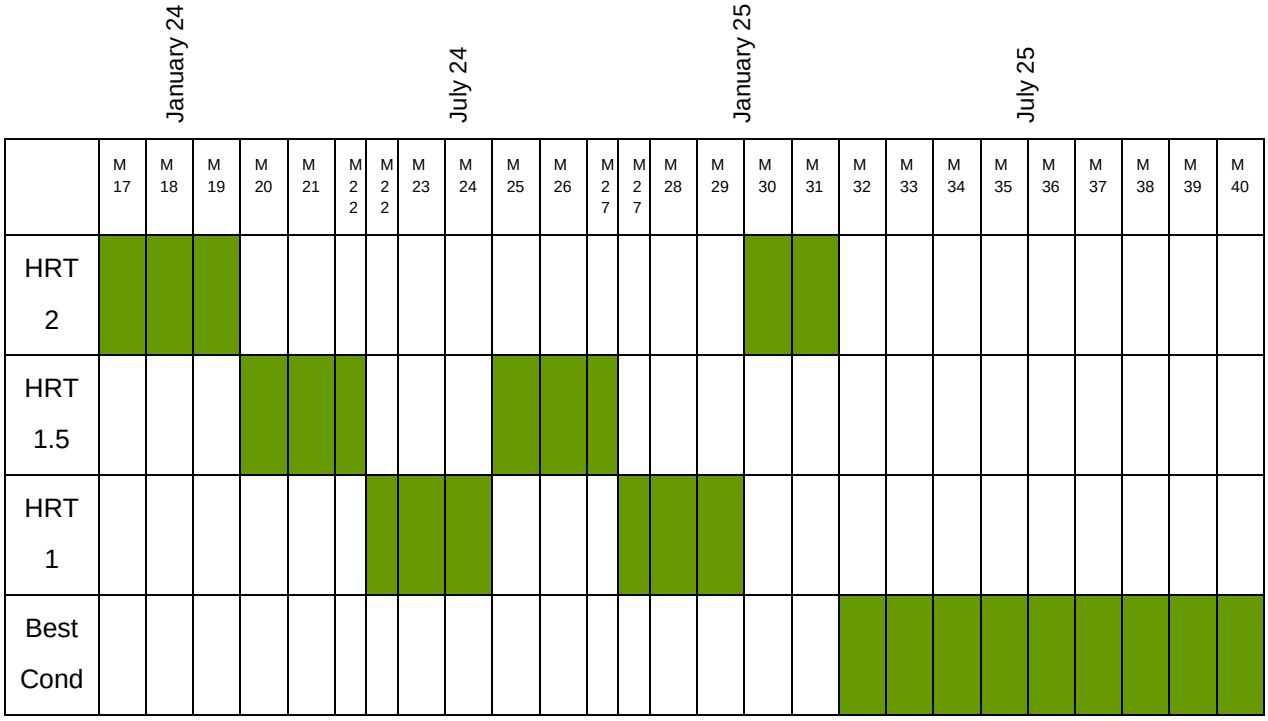

Figure 5. Scheme of the monitoring plan for the optimization of the BIODAPH system in Spain

4.2 Testing and optimization of the BIODAPH system in Greece

After the initial operation (start-up) of the BIODAPH reactor, the next phase is aimed at testing and optimizing the system. The testing consists of five operation phases in which three HRTs equal to 0.4, 1 and 2 days will be tested in order to optimize the operational conditions, taken as the meeting of the discharged effluent quality parameters regarding water temperature, pH, dissolved oxygen, and turbidity (Table 2). The number of *Daphnia magna* individuals will also be

counted. The monitoring control will include the measurement of standard wastewater parameters as COD, BOD_5, TSS, ammonium nitrogen (NH_4-N), phosphate (PO_4-P), in addition to water temperature, pH, dissolved oxygen, turbidity (which are continuously monitored by the MCU), in compliance with the Greek legislation for reuse of treated water in agricultural irrigation (Common Ministerial Decision KYA 145116/2011). Measurements will be carried out once every 15 days.

In addition, the monitoring control will include the measurement of microbiological quality (*Escherichia coli* and coliforms) parameters in compliance with the Greek legislation for reuse of treated water in agricultural irrigation (Common Ministerial Decision KYA 145116/2011). Measurements will be carried out once every month.

In each trial period it should be considered that the system needs of a period of stabilization for each HRT to reach the equilibrium conditions inside the reactors and the acclimatization of Daphnia which is at least a two-week period. Within this period, the maximum length of the individuals will be reached therefore the filtration of particle may be maximized. Each operational period will be implemented also for different climatological stations.

The test plan will then involve 30 samples that will be collected from January 2024 (M18) to November 2025 (M40).

To monitor emerging contaminants and pathogens, four sampling campaigns with five replicates per sampling campaign will be performed. Water samples will be sent either to the CSIC laboratory for the measurements of the pharmaceuticals and ARGs and to the UDG laboratory for the measurement of PFAs and microplastics.

Table 2. Monitoring plan for the optimization of the BIODAPH system in Greece.

Period	Actions
January 2024 -July 2024 (winter/ summer with the same HRT)	**Monitoring of the reactor at HRT=2 d** Monitoring of water temperature, pH, dissolved oxygen, turbidity, COD, BOD_5, TSS, ammonium nitrogen, phosphate Total Coliforms, E.Coli Counting of *Daphnia magna* individuals Pharmaceuticals, ARGs, PFAs and microplastics.
August 24-February 25 (summer /winter with the same HRT)	**Monitoring of the reactor at HRT=1 d** Monitoring of water temperature, pH, dissolved oxygen, turbidity, COD, BOD_5, TSS, ammonium nitrogen, phosphate Total Coliforms, E.Coli Counting of *Daphnia magna* individuals Pharmaceuticals, ARGs, PFAs and microplastics.
March 25- November 25 (winter/summer with the same HRT)	**Monitoring of the reactor at HRT=0.4 d** Monitoring of water temperature, pH, dissolved oxygen, turbidity, COD, BOD_5, TSS, ammonium nitrogen, phosphate Total Coliforms, *E.Coli* Counting of *Daphnia magna* individuals Pharmaceuticals, ARGs, PFAs and microplastics.

4.3 Testing protocols

Table 3. Standard water quality parameters

Parameter	Abrv. (Unit)	Methodology	Notes
Suspended Solids	TSS (mg/L)	Standard Methods 2540 D. Total Suspended Solid Dried at 103-105ºC (*)	Filter used in gravimetric analysis to be 1.2 µm & 0.45 µm pore size. Minimum of 2 samples. May also be determined by SS probe but regular gravimetric SS determination required for validation.
Conductivity	(µS/cm)	Standard Methods 2510 B. Electrical conductivity method (*)	
Turbidity	(NTU)		Nephelometric Turbidity Unit
Biochemical Oxigen Demand (5 day)	BOD_5 (mg O_2/L)	5210 b. 5-Day BOD Test / Hach Method 8043 (*)	Determination of dissolved oxygen before and after incubation at 20ºC ± 1ºC in complete darkness. Inhibition of nitrification must be employed.
Chemical Oxigen Demand	COD (mg/L)	5220 Chemical Oxygen Demand (COD) / Hach Method 8000 (*)	
Dissolved Oxygen	DO (mg/L)	DO probe	

*APHA, W. AWWA (2013). Water Environment Federation Standard Methods for the examination of water and wastewater 22nd Edition. Amer. Pub. Health Association.

Parameter	Abrv. (Unit)	Methodology	Notes
Ammonium	NH_4-N (mg N/L)	Ammonium Probe	
Total Nitrogen	TN (mg N/L)	Standard Methods 4500-N Persulfate Method / Hach Method 10072 (*)	Can alternatively be derived from TKN, nitrate and nitrite results
Total Kjeldahl Nitrogen	TKN (mg/L)	Standard Methods 4500-NORG Nitrogen (Organic) / Hach Method 8075 (*)	Proposed method: D block digestion
Nitrate	NO_3-N (mg N/L)	IC / Nitrate Probe / Hach Method 8039 (*)	
Nitrite	NO_2-N (mg N/L)	IC / Standard Methods 400-NO2 Nitrite / Hach Method 8507 (*)	
Total Phosphorous	P (mg P/L)	Standard Methods 4500-P. Methods C and D/ Hach Method 8190 (*)	
Total Ortophosphate	PO_4-P (mg P/L)	IC/ Standard Methods 4500-P. Methods C and D/ Hach Method 8048	
Faecal Coliform	(cfu/100mL)	Standard Methods 9221 E A-1 Broth Medium (*)	Colony forming units per 100 millilitres
Total Coliforms	(cfu/100mL)	Standard Methods 9222 B Membrane filtration (*)	Colony forming units per 100 millilitres

*APHA, W. AWWA (2013). Water Environment Federation Standard Methods for the examination of water and wastewater 22nd Edition. Amer. Pub. Health Association.

Sedimentation of particles in the reactors

Mass sediment concentration of particles will be measured through the volumetric concentrations of suspended sediment (in $\mu L \cdot L^{-1}$), which in turn will be analysed using the LISST-100X (Laser In-Situ Scattering and Transmissometry, Sequoia Scientific, Inc, Bellevue, WA) particle size analyser. The LISST-100X consists of a laser beam and an array of detector rings of progressive diameters which allow the light received at the scattering angles of the beam to be analysed. The device measures particle volume concentrations for 32 size-classes, (logarithmically distributed in the size range of 2.5-500.0 μm), using a procedure based on the diffraction theory of light. The LISST-100X has been found to perform well when determining particle size distribution and concentration for both organic and inorganic particles suspended in water. The D50 can be determined from the sediment distribution of particles.

Sedimentation rates in the inlet tank and the main BIODAPH compartments will be estimated by deploying sediment traps at each tank subsection (for 24 h in the inlet compartment and 48 h in the main compartment) 4 times/year. To collect settling material, six 0.1-m-long Pyrex test tubes with an 0.02-m diameter will be positioned (0.2 m and 0.5 m above the bottom tank). The collected samples (with 3 replicates) will be filtered with glassfiber filters previously weighed and dried at 60 °C for 24 h to determine the grams of solid mass deposited per area and time, at each location.

Daphnia counting

A sample of 2 litres from the upper layer of the main body of the BIODAPH reactor must be collected at two different positions across the surface of the reactor (1 L for each position). Water has to be gently poured through a mesh of 0.5 mm of pore diameter. As the water is poured *Daphnia* individuals will be retained by the mesh. The number of *Daphnia* retained by the mesh can be then counted. Be careful of not collapsing the mesh, which could make the counting difficult. After counting, *Daphnia* individuals should be returned back to the reactor as gently as possible. This last procedure can be repeated as many times as required until the 2 L of the water sample have been completely filtered. From the counting, the number of *Daphnia* per litre can be obtained.

Biofilm testing

The **biological status** of the biofilm on the lamellas of the reactor will be assessed during the operational period to establish the most appropriate conditions to obtain the maximum nutrient removal. To this end, structural and functional analysis of biofilm will be undertaken. The photosynthetic efficiency

and the phototrophic community composition of the biofilm will be measured in-situ. In addition, samples of biofilm for Chlorophyll-a (Chl-a) analysis to estimate the overall algal biomass and total biofilm biomass (hetero- and autotrophic biomass) will also be collected.

The **phototrophic community composition** will be measured in situ using the BenthoTorch portable fluorimeter probe (bbe Moldaenke, Schwentinenta, DK). This measurement is not invasive and determines the community composition of algal groups by means of the fluorescence excitation. This measurement is undertaken by seven diodes (LEDs) that emit light at three wavelengths to detect cyanobacteria, green algae, and diatoms (470, 525 and 610 nm, respectively). An additional LED of 700nm is used to compensate the effects of background reflection. The BenthoTorch measures the resulting fluorescence of Chlorophyll-a emitted at 680nm. The calculation of the respective biomass of the photosynthetic groups is via algorithm based on the fluorescence response to all different excitation wavelengths. The calculation algorithms are based on characteristic spectral fingerprints (fluorescence spectral signature) for each photosynthetic group. They will be used for real-time measuring of the main photosynthetic groups' densities (diatom, cyanobacteria, and green algae) of biofilm communities. Measurements will be done directly covering the colonized lamella surface with the probe outside the microcosm to determine the concentration of the main photosynthetic groups in µg chl-a/cm^2

The **photosynthetic efficiency** will be measured by means of a portable pulse amplitude modulated (PAM) fluorometer (Mini-PAM-II, HeinzWalz, Effeltrich, Germany). This measurement is not invasive and determines the biofilm functionality by means of the fluorescence excitation. The measures will be performed in situ by placing the light-emitting diode sensor at a constant distance from the biofilm growing in the lamellas. The Mini-PAM gives information about the minimum fluorescence yield that can be used as an estimation of the algal biomass and the yield. Yield is a measure of how photosystems perform in electron transportation and therefore of the capacity and performance of the photosynthesis process. Yield indirectly indicates the "health" status and autotrophy of biofilm.

The **Chlorophyll-a concentration (Chl-a)** will be used to estimate the algal biomass in biofilm. For Chl-a analysis, samples of biofilm will be taken from lamellas (known surface area). Once in the laboratory, Chl-a will be extracted from the biofilm using 90% acetone. Chl-a concentration will then be determined by spectrophotometric measurements (NanoPhotometerTM P-360) at 430 (carotenoid peak), 665 (Chl-a peak) and 750 (turbidity peak) nm.

Total biofilm biomass will be measured as ash-free dry mass (AFDM). This measurement accounts for the total organic matter contained in the biofilm and the referred to surface area. For this, a known area of biofilm is scraped from the lamellas, suspended in a known volume of water, and then filtered through a GF/F

Whatman glass-fibre filters. The dry biomass in these filters will be determined by measuring the mass difference before and after drying the filters at 60°C for 72h, the total organic content will be calculated by subtracting the remaining mass after combustion (4h at 500°C) from the dry biomass.

Emerging contaminants

Detection and quantification of ARGs. Water samples will be filtered through 0.22-µm-pore-size membranes (Isopore polycarbonate, Merk Millipore co., Germany) and filters will be stored at -20 °C until DNA extraction. They will then be processed using the DNeasy PowerSoil Kit (Qiagen Laboratories, Inc.) to obtain a final elution volume of 70 µL. The concentration and quality of the DNA will be tested using a NanoDrop Spectrophotometer 8000 (Thermofisher Scientific, Inc). High-capacity quantitative PCR arrays will be used on each extracted DNA sample to identify the abundance of clinically relevant antibiotic resistant genes (ARGs) that are resistant to tetracyclines, sulfonamides, macrolides, ß-lactams, quinolones, and aminoglycosides among others (He et al., 2020).

Detection and quantification of pharmaceuticals. 100 mL of water samples will be filtered through GF/F glass microfibre filters (Whatman, 0.7 µm), adjusted to a pH of 2-3 and spiked with 100 µL of EDTA 0.1M. All samples will be spiked with deuterated internal standards. Subsequently, the filtered samples will undergo solid-phase extraction (SPE) using cartridges (OASIS HLB, 200 mg, 6 mL) that are pre-conditioned using 6 mL of MeOH and 6 mL of H2O (pH=2-3). The cartridges will be stored at -80ºC until analysis (the storage time will always be less than 2-3 months). The cartridges will be eluted with 6 mL of MeOH and the solvent will then be evaporated to dryness at room temperature and reconstituted with 0.5 mL of water. The eluted samples will then be injected into a UPLC-QToF Impact II instrument (Bruker Daltonics, Bremen, Germany) for the detection and quantification of diclofenac, ibuprofen, triclosan, sulfamethoxazole, trimethoprim, tetracycline, ciprofloxacin, and erythromycin.

Detection and quantification of PFAs. Wastewater samples collected in polypropylene bottles will be filtered through glass-fibre filters with 0.7 µm pore size and analysed according to modified EPA draft method 1633. PFAS are quantified using the relative response of analytes to their respective isotope-labeled surrogates. Duplicate aliquots (250 mL each) of each sample are spiked with isotope-labeled surrogate mixtures and submitted to SPE extraction using Strata PFAS (200mg WAX/50 mg GCB/6mL) cartridges. SPE cartridges will be preconditioned with 5 mL 0.3% ammonium hydroxide, 5 mL methanol, rinsed with 5 mL ultrapure water and dried under vacuum. Elution is performed with 2x5 mL 0.3% ammonium hydroxide in methanol. Extracts are evaporated to dryness and reconstituted to 500 µL with methanol/water (95:5) and submitted to analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Analysis of microplastics. Influent and effluent water from the reactor is pumped into stainless-steel sieves with 1 mm, 355 µm, 100 µm and 20 µm meshes using a custom-made pumping station equipped with a flowmeter to measure the sample volume. The 355 µm sieve is visually examined to count the microplastics. Sieves of 100 and 20 µm are rinsed with 50 mL of MilliQ water. The rinse is treated to oxidize organic matter and remove inorganic particles, if present, and then filtered through a 1 µm glass filter. Microplastics are detected and counted, classified by size and shape, through a stereomicroscope with 10x magnification. Additionally, filters are analysed by Pyrolysis–gas chromatography–mass spectrometry (Py-GC-MS) to identify and determine the mass concentration of the polymers in the microplastics. Twelve polymers are targeted by Py-GC-MS: polyethylene (PE), polystyrene (PS), polypropylene (PP), polyvinylchloride (PVC), polycarbonate (PC), polyurethane (PU), nylon-6 (N-6), nylon-6,6 (N-66), polyethylene terephthalate (PET), polymethyl methacrylate (PMMA), styrene-butadiene rubber (SBR), and acrylonitrile butadiene styrene copolymer (ABS).

Metals and metalloids

Samples are acidified with nitric acid at pH<2 with nitric acid, left for two days and subsequently filtered through 0.45 µm cellulose acetate filters. Metals are determined by inductively coupled plasma mass spectrometry (ICP-MS), measuring ^{53}Cr, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{111}Cd, ^{118}Sn, and ^{208}Pb isotopes. ^{205}Tl and ^{103}Rh are used as internal standards.

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ANNEX I

Cultivation of *Daphnia* in the laboratory

The key to avoiding population fall-off/crashing is to have constantly favourable conditions, and to avoid sudden changes, such as large temperature drops, culture fouling, or the presence of toxic chemicals in the water. The *Daphnia* culture can be maintained for the longest time periods at temperatures between 19 and 21 °C.

Do not wash your hands with soap/detergent before you put your hands in a *Daphnia* culture unless you have thoroughly rinsed them afterwards as soap and detergents are toxic to *Daphnia*.

Do not overfeed *Daphnia* individuals to avoid fouling and toxic build-up of ammonia.

Prevent algal blooms since they tend to raise pH to very high levels (over pH 9) and coupled with a low ammonia concentration creates toxic condition for the *Daphnia*

Do not use airstones in a *Daphnia* laboratory culture. Use an open airline tube or a bio-foam filter (the latter contains an airstone inside in the apparatus, but the bubbles are not so fine as to harm them when they emerge into the tank).

Protocol for *Daphnia* cultivation, maintenance and collection

1. *Daphnia* collection and cultivation

Daphnia individuals can be either collected in a lake (natural or wastewater treatment ponds) or bought through Internet, since they are also used for feeding fish. The objective of *Daphnia* cultivation is to develop a numerous and strong population. Daphnia populations coming from wastewater treatment ponds do not need to be acclimated to the chemical conditions and if they are inoculated at warmer or colder temperatures out of the optimal range this can cause a drop of the population if the temperature is above or below 5 degrees apart from the optimal range. However, for smaller temperature shifts they are expected to acclimate.

Once collected, *Daphnia* can be kept and cultivated in water containers in the laboratory as shown below. The system can include a gentle continuous air flow

(using an aquarium air pump, for example). The air flush should be gentle, since *Daphnia* does not like turbulence. The place of testing should have hours of light and hours of darkness, since *Daphnia* has a daily pattern based on natural light.

Introduce *Daphnia* individuals in a container with mineral water (or treated wastewater) (Serra et al. 2021b, 2021c). They must be fed twice per week with, for example, a mixture of Spirulina powder and yeast. Introduce the Spirulina powder (¼ of teaspoon) and ¼ of teaspoon of yeast in a beaker of 1L and mix it vigorously until you obtain a homogeneous suspension. Then, wait for large particles to settle (for around 1 hour). Use the supernatant to feed the *Daphnia* culture. Since water is not continuously recirculating in a laboratory culture, an external air supply may be necessary to maintain the oxygen level in the system. The water in the container will need to be partially renewed periodically. The amount will depend on the volume of water and the number of *Daphnia* in the culture. Usually, the frequency recommended is once per week. Do not change too much water from the tank at once, a maximum of a 1/3 of the total volume is recommended.

Water in the containers should be maintained in an optimal range of temperature between 19 and 21 °C since within this range the capacity of filtering is at each maximum. For temperatures of 14.5 and 24 °C, the filtration capacity is reduced to half while the survival I still high. For temperatures higher than 28°C both filtration and survival are highly compromised. In any case temperatures should never be above 29/30°C. At such high temperatures the whole population can collapse (to death) in four days. *D. pulex* seems to do well at well at almost any temperature above 10 °C.

Daphnia cultivation

In order to cultivate *Daphnia*, it is recommended to fill the containers with *Daphnia* and water. Different kinds of water can be used:

- Mineral water or river water:
    - I) Inoculation: Prepare water with 0.1 $g \cdot L^{-1}$ spiruline (commercially available).
    - II) Operation: Renew 10% of the water twice a week. On Monday (or first day of the week) renew the 10% with raw mineral or river water. On Thursday (or 2nd day of the week) renew with mineral or river

water spiked with spiruline needed for getting 0.1 $g \cdot L^{-1}$ spiruline in the whole container. If you see that the container is still green before the 2nd renewal, renew with mineral water instead of adding more spiruline.

- Tap water:
    - The procedure would be similar to the described for mineral or river water. However, tap water should be kept during 48 h before being used to allow chlorine evaporation, since chlorine could be toxic for *Daphnia*.
- Secondary effluent of urban wastewater treatment plant:
    - The procedure would be similar to the described for mineral or river water. In this case, it is not needed to add spiruline to the media, since secondary effluent wastewater already contains bacteria and solids for *Daphnia*.

The tracking of the *Daphnia* system can be easily performed by visual inspection. Two main organisms will grow:

- *Daphnia*:
    - Normal growth/behavior: *Daphnia* are big enough to be seen by naked eye. Normally you will see them swimming along the container. They have a monthly pattern; thus it is normal that the population and the size of *Daphnia* can change along the month.
    - Issues:
        - ***Daphnia* color:** Normally *Daphnia* are grey/transparent. However, if the oxygen concentration is low, they start to produce more hemoglobin, so their color will turn into red. There is no need for worrying about it, they can survive at very low O_2 concentrations (0.5 $mgO_2 \cdot L^{-1}$). However, if this persists over time, consider reducing the amount of spiruline added to the system, or install an aquarium air pump.
        - ***Ephippia/resistance eggs*:** *Daphnia* normally reproduce through parthenogenesis. Parthenogenesis is the ability to self-replicate without fertilisation of any form (a type of asexual reproduction) - the offspring are exact genetic replicas of the parent (clones), and any differences in the physical state of the clones is due to environmental conditions. In the freshwater ecosystems (lakes and ponds), during the late spring, summer, and early autumn (northern hemisphere), *Daphnia* might reproduce by parthenogenesis, and in the laboratory, under optimal conditions they can undergo constantly yearly reproduction. On average, ten live young per individual (the entire race is made up of females during this period) are the result of parthenogenesis. Developing embryos are often visible in the mother's body

with no need of a microscope. Generation after generation of females can be born in this way, with new females reproducing as early as four days old at intervals as often as every three days, for up to twenty-five times in their lifetime. However, when the environmental conditions get worse, e.g., they feel that the temperature in the conditions is not the optimal or that the food in the containers is scarce, they produce resistance eggs, which are able to survive on bad conditions. These eggs are black, and, in our case, they might indicate that a more intensive water renewal is needed or that the optimal conditions should be re-established. When conditions improve again, the egg producing generations begin producing live young once again (all females), and the male sex dies out completely until it is needed when conditions worsen once again. For the new generation of *Daphnia* to grow from the eggs, in the laboratory it is necessary to keep the eggs in constant optimal temperature for about 10 days in fully dark conditions.

- There are often pulses of population growth in the laboratory containers if conditions are well maintained, when numbers increase almost logarithmically by parthenogenesis, using up a lot of food and causing overcrowding. When overcrowding the numbers might fall sharply and ephippia are produced again. In this condition a new container can be established with removing individuals from the overcrowded containers. The pulses will be clearly noticeable. In the laboratory, overcrowding, might produce a limiting size characteristic length scale of the individuals, which might be of a reduction of 25% comparing to the field characteristic maximum length scale of the individuals.
- Generally, the **life span increases as temperature decreases**, due to lowered metabolic activity. The average life span of *D. magna* is about 40 days at 25 °C and about 56 days at 20 °C. The average life span of *D. pulex* at 20°C is approximately 50 days.
- ***Chemicals*:** Daphnia are very sensitive to chemicals like the chloride or fluoride in tap water, which are extremely toxic to daphnia, even more so than to fish. They are also sensitive to metal ion concentration, like sodium, potassium, magnesium and calcium, which in increased concentrations can cause immobility and death, and daphnia are extremely sensitive to copper, zinc and most dissolved toxins (e.g. dichromate ions). Also they are sensitive at ammonium concentrations above 30 ppm and nitrite concentrations

above 2 ppm. Among metal containers, stainless steel is the safest because many metals will react slowly with water over time (e.g. Aluminium oxidises to form a skin of aluminium oxide, but small amounts of aluminium are released into the water). Plastic containers are the best.

- A **pH** between 6.5 and 9.5 is acceptable, with the optimum being between 7.2 and 8.5.

- ***Light conditions*:** Put the containers in 12 (light)/12 (dark) period of light span or a 16 (light)/8 (dark) light span. Avoid lamps that might secondarily heat the water in the containers.

- ***Stressors.*** Daphnia population can drop when they are under several stresses. Therefore, for the inoculation it is recommended that temperature keeps in the optimal range when the Daphnia are introduced in the system with wastewater. The wastewater has chemicals that already represent a stressor for them. With time Daphnia will adapt and they will cope with the natural change in temperature. Probably, when they are under different simultaneous unfavourable conditions their population will drop but it will recover by itself when the stress is over.

  If Daphnia in the system are submitted any time to different simultaneous stressors (chemicals, temperature, light) out all of them of the optimal range this can cause a severe drop of the population. The population drop will depend on the deviation of these stressors from the optimal conditions already defined.

  The presence of microplastics in the water represents another stressor. The greater the percentage of microplastics the worse. For percentages of microplastics above 50% respect to the percentage of food (particles ingested by Daphnia that bring nutrients for their living requirements) the Daphnia population will also drop. Percentages of microplastics below the 50% are not expected to produce a lethal effect on Daphnia but will cause a decrease in the efficiency of the system. However, microplastic percentages below 25% are not expected to produce changes in the Daphnia performance.

- Bacterial/algal biofilm:

- Growth: On the wall of the container a biofilm should grow. This biofilm will be in charge of depleting the nutrient content and it will be also used as a feed for *Daphnia*.
- Issues: The main issue related to the biofilm is if it grows excessively. If the nutrient load is too high, it can promote the growth of filamentous algae, which will deplete the dissolved oxygen, and it can seriously harm the *Daphnia* population. Filamentous algae should be removed manually from the containers.

Extra Maintenance considerations

- A *Daphnia* culture requires very little maintenance other than partial water changes (the amount really depends on the volume of water and the number of *Daphnia* in the culture - more water usually needs less changing, more daphnia usually means more water needs to be changed, to a maximum of 50% per week). Do feed your *Daphnia* on a regular basis, like Monday and Thursday.
- The key to avoiding population fall-off/crashing is to have constantly good conditions, and to avoid sudden changes, such as large temperature drops, culture fouling, or the addition of dangerous chemicals to the water.
- Do not wash your hands with soap/detergent just before you put your hands in a *Daphnia* culture unless you've thoroughly rinsed your hands because soap and detergents are toxic to *Daphnia*.
- Do not overfeed - if anything, underfeed the *Daphnia* to avoid fouling and toxic build-up of ammonia.
- Do not put *Daphnia* in a container of dense algae because algal blooms tend to raise pH to very high levels (over pH 9), and coupled with even a low ammonia concentration, this could be disastrous for the *Daphnia*, killing them in short order. Ammonia toxicity increases with higher pH.
- Do not use airstones in a *Daphnia* culture. Use an open airline tube or a bio-foam filter (the latter contains an airstone inside in the apparatus, but the bubbles are not fine enough to harm the *Daphnia* when the bubbles emerge into the tank).

2. Where to find Daphnia, locally.

Daphnia is an organism that is widespread around the world. In nature they are expected to be found in lakes, wetlands, but also in purification lagoons and constructed wetlands. Moreover, the usage of *Daphnia* as fish fed is growing, thus *Daphnia* can also be purchased in pet shops or shops specialized on aquariums (they can be even bought through Amazon website). There are approximately 150 known species in North America, and a similar number in Europe (many of these species are found on both continents, either through accidental introduction by man, or nature). Many foreign species have been

introduced to America and Europe from Asia and Africa (the most notorious of which is *Daphnia lumholtzi*, which is native to Africa).