# 1. Publishable summary

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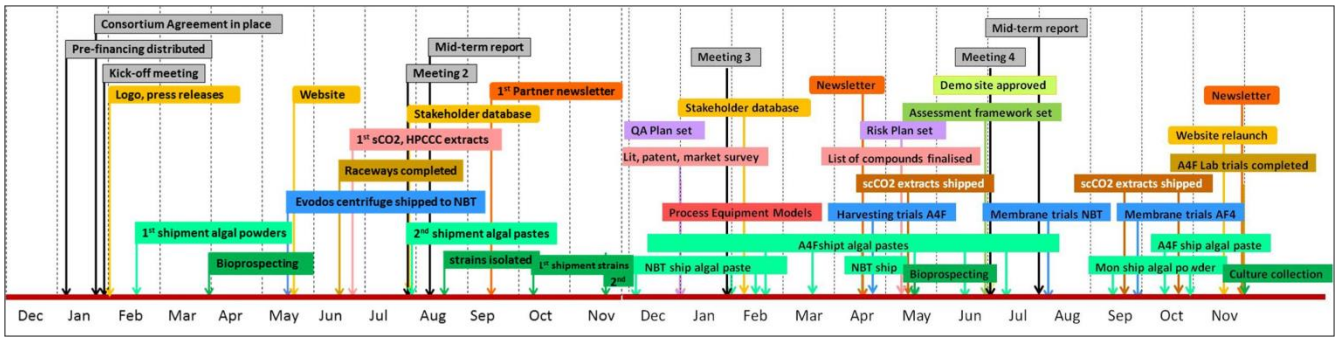
## 1.1. Project Objectives

**Context:** The D-Factory is a biorefinery which produces nutraceuticals, chemicals, feed and fuels from halotolerant microalgae, such as Dunaliella. Dunaliella grows in saline, non-potable water, around the globe. It captures $CO_2$ and harnesses sunlight energy, and naturally produces carotenoids, and other compounds of commercial value - bioactives, emulsifiers, polymers and glycerol. The D-Factory project will tune the algae to make these products in different proportions, then extract them using supercritical $CO_2$, HPCCC and membrane or chromatography, and formulate them to meet market requirements.

**The objectives of the project that will deliver the D-Factory biorefinery are as follows**:

1. Establish a D-Factory reference library of at least 12 new monoclonal halotolerant microalgal strains isolated either from partner/ stakeholder locations or developed by mutagenesis, cryopreserve where possible and make this available for D-Factory Partners to screen, characterise and use in the D-Factory demonstration of a microalgal biorefinery.
2. Establish production protocols by month 36 that enable suppliers of microalgal biomass to tailor their production of halophytic algae such as *Dunaliella* using techniques based on strain selection and/or cultivation, in order to meet biorefinery specifications.
3. Benchmark an open raceway facility producing kilogram quantities of *Dunaliella* biomass in terms of its technological, economic, environmental and social sustainability.
4. Develop and implement a pilot (10 $m^3$) cultivation PBR by month 24, which, by using controlled conditions,
    - Is capable of producing large quantities of high density (2.0 g/l DW) microalgal biomass tailored to meet D-Factory biorefinery demands,
    - Offers sustainable scalability to industrial scale (several hectares),
    - Provides data for assessment and modelling (by month 33 ),
    - Informs the creation of the D-Factory business plan (month 39).
5. Develop and demonstrate cell harvesting technologies to process ~2500l saline water /h and recover at least 90% intact *Dunaliella* cells at lowest cost from open ponds or PBRs by month 12 for the D-Factory demonstration
6. Develop methods for microalgal cell storage, handling and disruption (by month 6) and for microalgal bioprocessing using technology schemes based on $scCO_2$, HPCCC, and membrane technologies, to produce extracts/fractions for bio/chemical profiling, and bioactivity screening (month 24), and for D-Factory scale-up designs to industrial scale, taking into account technical, environmental and economic aspects using data obtained from mobile units installed at the D-Factory demonstration production site (month 48).
7. Confirm chemical structures on selected D-Factory biological materials, including by-products of microalgal processing, establish biological activity, measure product recoveries and purities, and identify any new compounds of interest (month 36), and develop product specifications for extracts, by month 48.
8. Draw up plans for adding value to *Dunaliella* extracts either through formulation, or through more rigorous purification for applications in pharmaceutical, nutraceutical, food, or cosmetic industries (for new business development), by month 46.
9. Establish a) conceptual designs for the construction and operation of a full-scale D-Factory demonstration facility, b) the basic functions of the high throughput platform and c) a thorough assessment of opportunities to improve materials and energy efficiency for the D-Factory demonstration facility, by month 36.
10. Provide a full sustainability assessment of the technological, environmental, economic and social sustainability including a SWOT analysis on strengths, weaknesses, opportunities and threats for establishing a *Dunaliella* production facility and D-Factory biorefinery for the production of named compounds from *Dunaliella*, including the sustainable management of by-products and wastes such as salt water by month 48.
11. Build an Innovation Platform to serve as an interactive forum able to capture the interests and activities of a wide range of stakeholders and integrate their local knowledge and experience with data from the D-Factory partners so that existing knowledge can be shared and new knowledge created.
12. Develop a comprehensive business case to show industrial investors the complete opportunity and scope for the creation of a *Dunaliella* microalgal bio-refinery and raise additional investment for the D-Factory demonstration.
13. Manage and disseminate information at the same time as protecting the continuing acquisition of IP.

## 1.2. Work performed Dec 2013-Nov 2015




- **D-Factory Biomass Production:** 22 new monoclonal strains of D. salina have been isolated from various locations in Israel and Spain, 6 strains distributed to partners, and preservation methods using cryogenics installed. A mutagen, 5-Bromouracil has been used to screen and select new mutants of *Dunaliella*.

  Cultivation parameters (light intensity and duration; salinity; temperature; and CO2 concentration in the extracellular medium) and strain type on pool sizes of key metabolites (glycerol, carotenoids) has been investigated. Growth of 1 strain has been studied in a green-wall flat panel (GWP) photobioreactor (PBR) and in a tubular PBR (TPBR) and different recirculation pumps, levels of oxygen accumulation and light intensity have been investigated to establish requirements for maintaining carotenogenic cultures.

  A raceway at NBT was refurbished to a state-of-the-art pilot plant using cost efficient designs. It informs Demo plant designs, harvesting technology development, a full sustainability assessment, and also supplies kg quantities of algae for downstream processing. Flowsheet designs anticipate preparation of a high quality inoculum in GWP (green wall panels), followed by a growth phase in tubular reactors (TPBR)) and finally the carotenogenesis stage and a first version of the pilot unit layout has been completed, along with the major definitions (number, type and dimensions of the cultivation units) and pilot unit expansion construction has started.

- **D-Factory Biomass Harvesting:** EVODOS T10 spiral plate technology has been implemented in batch mode configuration at NBT and A4F partner sites. Dunaliella cells have been harvested using Westfalia centrifugation technology, and the Evodos T10 and the technology has been benchmarked. Membrane ultrafiltration was investigated to pre-concentrate biomass before centrifugation. Construction of continuous mode harvesting systems is being finalised for factory, then on-site testing at NBT.

- **Shipment of algal samples for processing:** Kg quantities of spray-dried and freeze-dried algal powder as well as frozen algal paste harvested using Evodos technology have been shipped from NBT to partners for testing methods and developing analytical protocols. Algal pastes prepared from cultures maintained in GWP and harvested using Evodos technology have also been shipped by A4F to IBET, SP and UOG.

- **Biomass processing and formulation:** Shelf-life of samples and cell disruption methods have been reported. sCO2 and HPCCC technologies have been applied to Westfalia- and Evodos-harvested cell powder. HPCCC technology has also been applied to Evodos-harvested cell paste. Different solvent systems have been investigated to define purification protocols for lutein and zeaxanthin and carotene isomers. Membrane resistance to solvents has been investigated in readiness for developing processes to enhance purification steps. Enzyme assays for carbonic anhydrase, glycerol-transforming and redox enzyme content were progressed and peptide extracts prepared for further characterisation.

  Analytical protocols were defined and standardised between laboratories for quantifying protein, carbohydrate, lipid, ash, pigments and carotenoids. NMR and LC-MS methods were established along with assays to test anti-diabetic and anti-obesity activity and the cytotoxicity lab has been set up for efficient bioactivity screening of samples and positive controls have been purchased and evaluated.

- **Market research** has been undertaken in preparation for formulating preparations for customers and initial formulation trials have been initiated using ScCO2 extracts. Tests with standard antioxidants have led to the development of shelf life and recommended storage conditions. A literature, patent and market survey has been reported as well as a list of Dunaliella extracts/fractions that should be further developed / formulated for commercialisation.

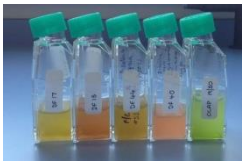
- **D-Factory Sustainability Assessment, Modelling Optimisation and conceptual plans for the Demo:** Definitions and settings along with determination of system boundaries have been set for sustainability assessments and several scenarios have been developed for evaluation. Three proposed scenarios of Dunaliella

biorefineries have been designed and integrated providing basic mass and energy balances. Based on these designs, energy and water analysis has been conducted to establish savings in their consumption.

- **Demo site & Business case** A suitable location for a demo was sought and costings at various algal plants carried out. Preparation of a business case is underway.
- **The Stakeholder Database and Innovation Platform:** **S**takeholders have been analysed and mapped to assign levels of most/least importance and influence to the D-Factory and now informs development of the communication plan.
- **Dissemination tools:** The project identity was formulated including the project logo, letterhead and presentation templates for lectures and posters. The website was created and launched. A brochure has been produced and newsletters produced. Multiple press releases and invited oral presentations have been made.
- **Scientific Co-ordination and Project Management:** Scientific co-ordination has been monitored and progressed by regular communication and minutes of meetings produced and stored in the Intranet of the Website. 5 project meetings of the steering committee and IP committee have been mounted. A Repository, Project Calendar and Helpdesk support partners. Internal Newsletters have been created since Sept 2014. The D-Factory **Resource Information Database** has been established and scientific and intellectual property databases are being maintained by the Project Officers. The Project management book, Quality Assurance and Risk Assessment Plans have been developed and the first risk and quality assessments conducted.

## 1.3. Main Results achieved so far

**D-Factory strains from saline waters in Eilat, Israel and Monzon, Spain:** The 22 strains of the D-Factory culture collection are well-preserved using cryogenic methods and genetic analyses are underway. Laboratory mutants developed using 5-Bromouracil, when tested outdoors were either unable to compete with prevailing natural strains (*D. salina* mutant) or to withstand environmental conditions (*D. parva* mutant). Three D-Factory strains of *D. salina* selected for characterization differ significantly in terms of carotenoid content; response to growth in ammonium; nitrogen depletion and light intensity. The globally-used *D. salina* standard, CCAP19/30 may in fact be a strain of *D. tertiolecta*. Cellular glycerol content correlated with cell volume, whilst the tendency for culture crash increased at temperatures >20ºC due to the greater rate of growth increase of bacteria. Biomass yield can be increased under continuous light with non-limiting CO2 but results in damage to photosynthetic and respiratory systems. Increased salt in the extracellular medium will increase glycerol yield but not for extended periods of growth, whilst the time of harvesting affects the yield of glycerol and starch.



**Cultivation in a Green Wall PBR and tubular PBR fence:** In pilot scale Green Wall (GW) and tubular PBRs, a D-Factory strain grew well in the green, non-carotenogenic stage, with improvements in yield afforded using non-refined seasalt media. However culture in the orange carotenogenic stage was not readily implemented and, amongst several factors investigated, the main causes were proposed to be due to improper medium chemistry and trace minerals free salt, insufficient control of radiation impinging on the reactors and the adhesive nature of the wall-less Dunaliella. Pilot unit expansion construction has started in the hope that it should prove possible to cultivate orange cultures in tubular PBRs in current trials.

**Raceway in Eilat working at scale:** NBT established a pilot raceway facility in Eilat using ideal materials and a cost-efficient design. It is in full operation, produces Dunaliella at scale and supports harvesting trials using Evodos technology. Data are being captured to inform construction of the future Demo plant and facility is well-placed to validate the concept of a microalgae biorefinery. Cultivation conditions have been optimised for carotenoid production as a result of the directions provided by partner reports. The facility has served as a benchmark of algae cultivation in a raceway, however control of predators remains a problem.

**Novel live cell harvesting using Evodos T10 spiral plate technology** has been implemented as a batch system at both NBT and A4F; a continuous harvesting design is in construction. Membrane ultrafiltration, before centrifugation, pre-concentrated biomass up to a concentration factor of 6 for green non-carotenogenic cells, but only 2 for orange cells which have a higher susceptibility to shear stress.

**Methods for microalgal cell storage, handling and disruption and biomass processing.** Algal pastes contain heterotrophic bacteria and are therefore best processed as rapidly as possible. Analytical protocols have been defined and standardised between laboratories for quantifying protein, carbohydrate, lipid, and ash as well as

pigments and carotenoids. Fast lipid, chlorophyll and carotenoid quantification using APCI-MS is a promising screening technique for optimising productivity within the D-Factory.

Enriched extracts of 9-cis β-carotene can be obtained using scCO2 and HPCCC methods, whilst detailed analyses of different extracts of peptides, enzymes and carotenoids now pave the way for an integrated process of purification for compounds of value. This is also informing process flow schemes and design of Demo facilities. The focus will move towards pharmaceutical screening increasing the likelihood of discovering novel patentable compounds. Promising results from anti-diabetic screening are informing new purification strategies to identify the active agent(s).

**Added value through formulation and more vigorous purification:** Studies of carotenoid stability of scCO2-extracts have been initiated using emulsified systems with water and food/pharma-grade surfactants. Tests of stability of the carotenoid during the emulsification process have been initiated.

**Demo site & Business case** Initial research looked at different coastal industrial salt locations in countries with an all year growing season, but these were ruled out in favour of a underground salt facility at Monzon, Spain, which met or exceed in several criteria: it has an onsite power plant emitting flue gas with ~6% CO2 from the combustion of natural gas, predator-free salt brine, water, energy and heat, available land, good communications and infrastructures, industrial environment, qualified staff and company commitment, as well as evidence for the growth of Dunaliella all year and no requirement for marine environment permits. Costings at various algal plants have been carried out for comparison with the selected Monzon facility and preparation of a business case is underway.

**D-Factory Sustainability Assessment, Modelling Optimisation and conceptual plans for the Demo:** First steps were taken to transform the assessment of generic scenarios into an assessment of the demo facility coming up soon. All specific tools to perform the sustainability assessment such as LCA are now developed, reviewed, implemented and ready to be used to assess all D-Factory scenarios, which includes especially the demonstration concept at Monzon and all technologies investigated in D-Factory. For the Demo, an optimization model for screening processing paths has been developed, but more reliable data is needed to provide realistic solutions.

**The Innovation Platform** Approximately 800 stakeholders have been analysed and mapped to assign levels of most/least importance and influence to the D-Factory in order to identify those groups that should be represented in the D-factory Innovation Platform. The analysis now informs development of the communication plan with details on how to communicate to these stakeholders and with what level of frequency. To support this activity, the newly redesigned website includes a knowledge base which will serve as part of the design for the Innovation Platform aimed at integrating stakeholder partnerships and activities.

**Dissemination tools have been developed:** The website has been launched and the project identity formulated through the use of the project logo, letterhead and presentation templates for lectures and posters. A brochure has been produced to outline the project and illustrate its progress. The D-Factory Poster has been presented at several scientific conferences and trade shows. Multiple press releases and invited oral presentations have been made at venues in Europe and internationally.

**Methods for Scientific Co-ordination and Management have been established:** Project progress is catalysed by regular communication, electronically and in person. Minutes of teleconferences and meetings are stored in the Intranet of the Website. Progress made by Partners is reinforced through internal Newsletters which illustrate day-to-day information transfer between partners. The Newsletter also deals with "Project Publications", "Recent Scientific Literature", "Competitor Intelligence" and "Upcoming Conferences". A Quality Assurance Plan and a Risk Management Plan is in place and has been executed in a first analysis by WP leaders.

## 1.4. Expected Final Results:

A sustainable D-Factory demonstration in Europe that sets **a world benchmark** for a microalgae biorefinery based on biomass from halotolerant and halophilic microalgae.

## 1.5. Potential Impact and use

Global adoption of licenced microalgae biorefinery processes utilising non-potable saline waters for the production of nutraceuticals, pharmaceuticals, protein and green chemical feedstocks.