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\textsubscript{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\textsubscript{2}, HPCCC and membrane and 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\textsuperscript{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), and
   - provides data for assessment and modelling (by month 33 )and
   - 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\textsubscript{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 2014

- **D-Factory Biomass Production:** Several viable strains of *D. salina* and other algae from various locations in Israel have been isolated and are being genetically typed. Three of these have been distributed to partners for characterisation and growth trials. One of these was cultivated in scale-up in a green-wall flat panel photobioreactor (PBR) and in a tubular PBR. A classical mutagen, 5-Bromouracil has been used to screen and select new mutants of *Dunaliella*.

Laboratory experiments aimed at documenting the effects of cultivation parameters (light intensity and duration; CO2 concentration and salt in the extracellular medium) on pool sizes of key metabolites (glycerol, carotenoids, lipids) were progressed. Carotenogenesis induction by cultivation under nitrogen-limited conditions was investigated.

In tubular PBRs different recirculation pumps have been tested for their effects on shear stress. A PBR-to-raceway process is now being developed. A first version of the pilot unit layout has been completed, along with the major definitions (number, type and dimensions of the cultivation units).

A redundant raceway at NBT was refurbished to a state-of-the-art pilot plant using cost efficient designs. The facility is being used to inform Demo plant designs (WP6, WP9) and a full sustainability assessment (WP7). It is also supplying kilogram quantities of algae to the partners for downstream processing.

- **D-Factory Biomass Harvesting:** EVODOS T10 spiral plate technology based on dynamic settling has been implemented in batch mode configuration at NBT and A4F partner sites. Cells of *Dunaliella* have been harvested from a raceway using Westfalia centrifugation technology, and the Evodos T10. Calculation of % recoveries of a cytoplasmic marker has enabled the existing cell-harvesting technology to be benchmarked. Construction of continuous mode harvesting systems is underway.

- **Shipment of algal samples for processing:** A 1st shipment of kg quantities of spray-dried algal powder for testing methods and developing analytical protocols was made by NBT in Feb 2014 using NBT strains of algae that were cultivated in pre-existing raceways owned by NBT and harvested using Westfalia technology. A 2nd shipment of kg quantities of frozen algal paste prepared using Evodos technology was made in Aug 2014 once Evodos technology was installed and the NBT pilot facility was completed.

- **Biomass processing:** sCO2 and HPCCC technologies have been applied to Westfalia-harvested cell powder. HPCCC technology has also been applied to Evodos-harvested cell paste. Methods are being developed to produce sufficient quantities of this material in a freeze-dried condition for sCO2 extraction. Enzyme assays for carbonic anhydrase and redox enzyme content were progressed.

Fast lipid, chlorophyll and carotenoid quantification methods were developed. Analytical protocols were defined and standardised between laboratories for quantifying protein, carbohydrate, lipid, ash, pigments and carotenoids. Harvested algal pastes were investigated for shelf life and cell disruption.

- **D-Factory Sustainability Assessment and Modelling Optimisation:** The methodological approach, which includes establishing the general definitions and settings along with determination of system boundaries, has been presented and discussed with all partners. Several scenarios have been developed for evaluation.

- **The Stakeholder Database and Innovation Platform:** The Stakeholder database was constructed and is now being populated and used to develop the business case (WP8) and Innovation Platform and the first set of data has been collected.

- **Dissemination tools:** The project identity was formulated: the project logo was created along with letterhead and presentation templates for lectures and posters. The website was created and launched. A brochure has been produced. The D-Factory Poster has been created and presented at several scientific conferences and trade shows. 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, both electronically and in person. Minutes of teleconferences and meetings have been produced and stored in the Intranet of the Website. 2 project meetings of the steering committee and IP committee have been mounted. A Repository and Project Calendar have been created to support partners and a project Helpdesk established. Webinars have been held on financial and contractual matters to support partners. Monthly 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, and plans for Quality Assurance and Risk Assessment have been developed.
1.3. Main Results achieved so far

- **Financial and Contractual Management systems are in place.** The main results achieved in year 1 are illustrated on a timeline in Fig. 1 and illustrated below.

![Timeline Illustration](image)

- **Raceway in Eilat working at scale:** NBT has established a pilot raceway facility in Eilat (WP2) through the efficacy of being able to convert 4 redundant raceways into D-Factory facilities. The facility used ideal materials and a cost-efficient design. It is in full operation and producing *Dunaliella* at scale (Fig. 2). It has been configured to support harvesting trials using Evodos technology (WP2) and enables data to be captured that will inform construction of the future Demo plant (D9.1) and a full sustainability assessment (WP7). The cultivation conditions are currently optimised for carotenoid production, but can be systematically altered to produce other high-value products depending on the findings of WP1. This is a substantial achievement, placing the Project in a position to produce and process algal biomass on demand. The facility is well-placed to validate the concept of a microalga biorefinery.

- **Cultivation in a green wall panel PBR and tubular PBR fence:** A4F has been able to successfully cultivate a D-Factory algal strain in a green wall panel PBR and tubular PBR (D1.4 ongoing – see Fig. 3). Culture viability remained high in simulated seawater that lacked several micronutrients. Carotenogenesis induction by cultivation under nitrogen-limited conditions was positive. A PBR-to-raceway process is now being developed to underpin large-scale *Dunaliella* production. This involves the development of state-of-the-art technology specially formulated for cultivating the fragile (cell wall-less) *Dunaliella* algae. A first version of the pilot unit layout has been completed, along with the major definitions (number, type and dimensions of the cultivation units).

- **Novel live cell harvesting using Evodos spiral plate technology:** Evodos T10 spiral plate technology based on dynamic settling rather than using Westfalia methods of centrifugation has been implemented at both NBT and A4F (Fig.4). The technology is now fully operational and *Dunaliella* cells after harvest have been shown to be intact and alive. Its implementation has enabled partners to receive D-Factory-specific cells containing both cytosolic and lipidic components for assessment and fractionation. It currently operates in batch mode and is now being developed for continuous harvesting.

- **Shipment of algal samples for processing:** Crucially for the project progress whilst D-Factory strains were being isolated and Evodos technology was being installed at NBT and A4F sites, a 1st shipment of kg quantities of algal powder for testing methods and developing analytical protocols was made available by NBT in February 2014 using NBT strains of algae that were cultivated in pre-existing raceways owned by NBT and harvested using Westfalia technology. This has provided invaluable insight into handling this type of biological material, engaged all partners in an active collaboration and afforded a biological benchmark for the project. A 2nd shipment of kg quantities of algal paste prepared using Evodos technology was made available in Aug 2014 once Evodos technology was installed and the NBT pilot facility was completed.
**First Purification strategy formulated:** DE and NATECO have succeeded in establishing a purification strategy using sCO\textsubscript{2} and HPCCC technologies to provide two enriched extracts tentatively identified as high-value carotenoids: lutein and 9-cis β-carotene (Fig. 3.5) (D3.2, D3.5, D5.3 ongoing). Excellent weight enrichment (50-100-fold) was achieved using HPCCC. Fast lipid, chlorophyll and carotenoid quantification using APCI-MS confirmed the presence of lutein/zeaxanthin and other carotenoids. The strategy will now be refined and optimised (D3.4 ongoing) to inform process flow schemes (WP6) and construction of Demo facilities. The focus will move towards pharmaceutical screening (WP4), increasing the likelihood of discovering novel patentable compounds.

**D-Factory strains from saline waters in Eilat:** New D-Factory strains have been isolated from waters in Eilat, a logical starting point for cultivation methods development. 3 of these have been shipped to partners. Preliminary comparative studies confirm differences among the strains with respect to doubling time, biomass yield and content of carotenoids, chlorophyll and glycerol. Laboratory mutants that were developed using a classical mutagen, 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).

**Laboratory cultivation tests for optimising yields of biorefinery products:** Laboratory cultivation studies showed that biomass yield can be increased under continuous light with non-limiting CO\textsubscript{2} but since photosynthetic and respiratory systems are damaged by growth in continuous light this is not recommended for continuous culture. Increased salt in the extracellular medium will increase glycerol yield but not for extended periods of growth, whilst the time of harvesting may impact on the composition of cell carbohydrates (glycerol or starch). Laboratory experiments aimed at documenting the effects of cultivation parameters on pool sizes of key metabolites (glycerol, carotenoids, lipids) are underway.

**Detailed biochemical work:** Harvested algal pastes have been investigated for shelf life and cell disruption. Algal cell disruption is effective by freeze-thaw. Freezing cell pastes causes algal cells to burst but fungal mycelia and bacterial cells remain intact. These and associated analytical data have led to the development of a methodology to transfer large frozen samples from NBT. A solution of ~30% wt glycerol in water may be suitable preservative of fresh harvested cells. 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.

**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 (>10) and invited oral presentations (>15) have been made at venues in Europe and internationally.

**The Stakeholder Database has been constructed:** The Stakeholder database is now being populated: it currently contains details of >600 stakeholders. In due course the contacts in the database will support research for conducting the Social Assessment (WP7) and for establishing the Innovation Platform and enabling a specifically targeted approach to support activities associated with the Business Case (WP8) and Demo (WP9).

**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.

### 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 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.

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**Figure 5:** (a) Dunaliella cell powder after sCO\textsubscript{2} extraction; (b) extract prepared by sCO\textsubscript{2}; (c) HPCC chromatographic separation of Dunaliella extracts