Nutritional and Health-Related Environmental Studies (NAHRES)

Application of Stable Isotope Techniques in Environmental Enteric Dysfunction Assessment and Understanding its Impact on Child Growth

Brief Summary

Retarded linear growth, widely referred to as stunting, is rampant in low and middle income countries, affecting a total of 161 million children under the age of five years; it develops in the first 1000 days of life, and becomes irreversible if no appropriate interventions are in place. Environmental Enteric Dysfunction (EED) is the presence of diffuse, upper small bowel villous atrophy accompanied by the presence of morphologic evidence of barrier disruption and inflammation. EED affects presumably 50-95% of all children under the age of 5 years in resource poor settings. Retarded growth, altered gut microbiota, and decreased vaccine responsiveness are considered the most important consequences of EED and are attributable to: altered intestinal structure and function, defects in nutrient absorption, reduced growth hormone activity, altered host immunity and change in microbiota composition and diversity. Despite the significance of EED to infant and child nutrition and health, biomarkers and simple diagnostic techniques for the definition and classification of EED are lacking. This CRP aims to validate and apply a novel, non-invasive stable isotope technique (^{13}C Sucrose Breath Test) to foster a better understanding of pathways underpinning EED and child growth. The key results of the CRP will be: 1) non-invasive ^{13}C Sucrose Breath Test to diagnose EED and assess its effects on health; 2) improved technical capacity to diagnose and assess health of EED populations; 3) new data on the pathways underpinning the relationship between EED and child growth including dietary, mucosal integrity/permeability and nutrient and energy partitioning. The ultimate outcome will be a better understanding of the relationship between EED and child growth which will in turn contribute to the development of diagnostic tools for EED to facilitate its prevention, treatment and management to ensure good health.

Background and Situation Analysis

Introduction

A large proportion of the population in low- and middle-income countries (LMICs) still live in environments characterized by poor water, sanitation and hygiene (WASH) conditions. According to the WHO and UNICEF, an estimated 2.5 billion people remain without access to improved sanitation facilities (2015)[1]. In addition, many LMICs continue to struggle with a high burden of undernutrition, demonstrated by the nearly 805 million undernourished people worldwide, the vast majority of whom live in LMICs (2012-2014)[2]. Stunting, defined as height-for-age z score less than -2 standard deviations of the World Health Organization Child Growth standards develops in the first 1000 days of life, and becomes largely irreversible if no appropriate interventions are in place. Stunting affects 159 million children under the age of 5 years, representing a global prevalence of 23.8%[3]. The consequences of stunting include increased infant and child mortality and morbidity; increased risk of overweight, obesity and non-communicable diseases later in life; and low psychomotor development and lost economic potential[4]. Inadequate nutrition and recurrent infection are the primary drivers of stunting. However, evidence now shows that all known nutritional interventions combined may only partially

prevent stunting[5]. Poor hygiene and absence of adequate sanitation may play a role but evidence to support a causal relationship is largely lacking. Living in poor sanitary conditions may induce gut dysfunction[6,7], referred to as environmental enteric dysfunction (EED).

A technical meeting on environmental enteric dysfunction (EED) organized and hosted by the International Atomic Energy Agency (IAEA) in Vienna, Austria from October 28–30, 2015 identified gaps including need for a clear classification and understanding of causal pathways underpinning EED. The meeting recommended the development of practical, simple, and affordable tools to diagnose and characterize EED to allow better targeting of interventions in vulnerable populations. Stable isotope techniques were recommended for the assessment of absorptive capacity/permeability of the gut, bacterial translocation and body composition as a proxy indicator of dietary quality and morbidity. A follow up technical meeting from 31 May-1 June 2016 identified three EED-related domains where stable isotope techniques are applicable , namely 1) dietary intake, including nutrient absorption; 2) microbial translocation [mucosal integrity/permeability and inflammation (gut and systemic inflammation)] and; 3) host metabolic response (e.g. growth and nutrient metabolism).

EED is the presence of diffuse, upper small bowel villous atrophy accompanied by the presence of morphologic evidence of barrier disruption and inflammatory infiltrates. EED affects presumably 50-95% of all children under the age of 5 years in resource poor settings. Retarded growth, altered gut microbiota, and decreased vaccine responsiveness are considered the most important consequences of EED and are attributable to the following potential mechanisms.

- ✓ EED adversely affects intestinal structure and function and is characterised by villi blunting, chronic gut and systemic inflammation, altered gut permeability, bacterial translocation and nutrient malabsortption[8].
- ✓ EED is linked to defects in carbohydrate[9], amino acid[10,11], fatty acid[12] absorption. Gut mucosal damage can affect lactase activity, thereby limiting the utilisation of lactose, the major carbohydrate in breast milk[13]. Some amino acids, especially glutamine, which are involved in maintenance of gut integrity show an overall lower concentrations in malnourished pigs compared with reference models[11]. Increased arginine catabolism without an increase in arginine flux has been observed in Indian women of childbearing age compared to their peers Jamaican and American women[14]. Pancreatic lipase activity is diminished in EED[12]. These effects are more pronounced in severe acute malnutrition (SAM) with kwashiorkor[9,12]. How much of altered nutrient metabolism is attributable to undernutrition on one hand and EED on the other remains to be investigated.
- ✓ Insulin growth factor (IGF-I) is diminished in EED, likely due to increased levels of systemic cytokines, cortisol and Insulin growth factor binding-protein that act in combination to inhibit protein synthesis and growth failure[15].
- ✓ Altered gut microbiota affects gut function, however, the causal pathways require further elucidation.
- ✓ EED affects host intestinal immunity, which could be enhanced by for example oral poliovirus vaccine[16].
- ✓ EED affects vaccine efficacy via reduced gut absorptive function, micronutrient deficiencies and pathogen-vaccine competition[17].
- ✓ EED is exacerbated by poor infant feeding practices such as early introduction of complementary foods11. Emerging evidence suggests that exclusive breastfeeding is protective against gut inflammation associated with EED[18].

Study Design

The CRP will be implemented in three phases namely: Phase 1 will be implemented by selected contract holders only. Prospective Research Contract holders will be eligible to apply for various aspects within the themes under Phases 2 and 3.

Phase 1: Protocol optimisation and validation of 13C SBT.

The existing ^{13}C Sucrose Breath Test is based on the tracking of natural enrichment of sucrose with ^{13}C. It is postulated that higher ^{13}C enrichment would be required to have this technique applicable in EED settings. Phase 1 will focus on the validation of ^{13}C SBT with two distinct but inter-dependent activities: 1) To optimise the protocol for ^{13}C-SBT and 2) to confirm that ^{13}C-SBT reflects intestinal sucrase/isomaltase activity by biopsy.

Phase 2: Cross sectional survey to compare the suitability of 13C SBT compared to combined L:R and K:T in EED diagnosis

All measurements will be done in a one off cross sectional survey. We will test the suitability of the ^{13}C SBT compared to combined L:R and K:T ratios among 100 infants 6-9 months of age living in a high risk population as defined by socio-economic status indicators: water, assets, maternal education and income index (WAMI). Infants will be declared to have EED (EED+ve) if they meet the criteria for both L:R and K:T ratios. Infants who do not meet the criteria for L:R or K:T or both will be deemed to be EED free (EED –ve). ^{13}C SBT will be assessed in both EED+ve and EED–ve infants to set ranges that will be used for the next phase. Additional data on weight, length/height, dietary intake, body composition and energy expenditure will contribute to understanding how EED impacts on nutrient intake and metabolism (reflected as body composition and energy expenditure) and the formulation of recommendations for energy and nutrient intake in the context of EED.

Inclusion criteria: All infants 6-9 months of age living in a population deemed to be high risk for EED will be eligible to participate in the cross sectional study. Children with severe acute malnutrition (SAM) and signs of chronic illness will be excluded as these are known to contribute to enteropathy.

Sample size: We will include a convenient sample of 100 infants. Due the absence of data on EED and stunting, a difference of at least +0.2 in LAZ between EED+ve and EED-ve groups will be considered to be biologically relevant. The WASH Benefits Study in Kenya and Bangladesh assumed a +0.15 difference in LAZ[25]

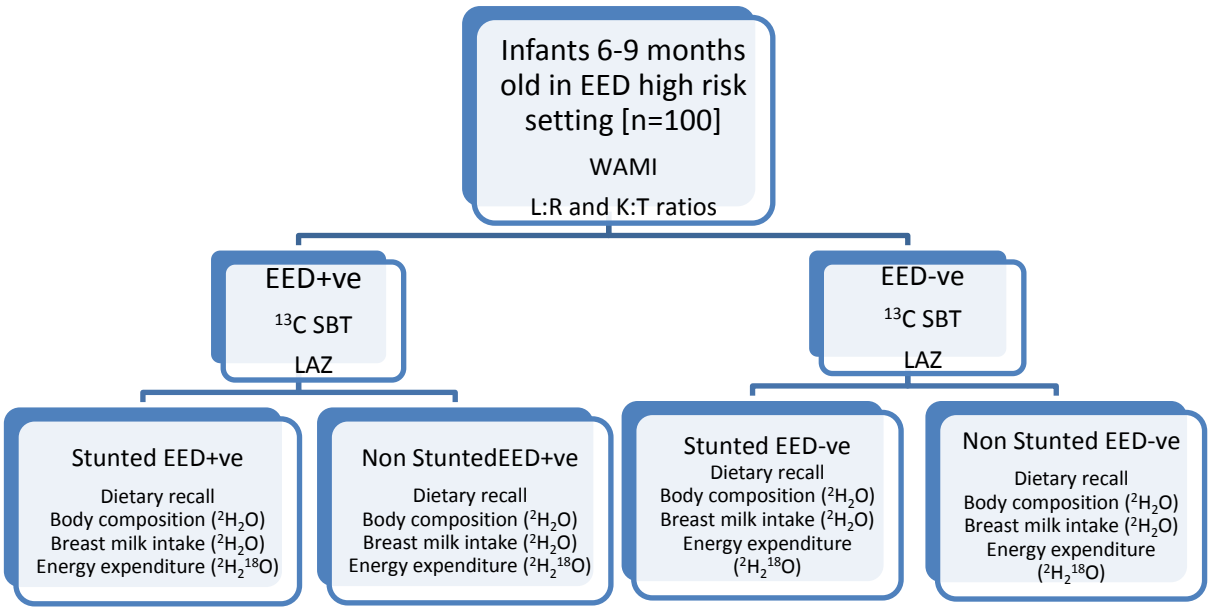

Figure 1: Proposed Flow Diagram for Phase 2

Phase 3: Prospective Cohort Study to understand the link between EED and child growth

This will be a prospective cohort study in which the primary question will be how EED affects linear growth trajectory in infants from 6 – 18 months of age. This phase will address pathophysiological mechanisms between EED and child nutritional status with specific focus on linear growth velocity. Additionally data generated will contribute to evidence on why some children with EED grow normally while others get stunted. A total of 200 non-stunted infants 6-9 months of age [100 from high risk and another 100 from low risk EED contexts] will be included and followed up for 12 months with quarterly measurements. The level of risk for EED at recruitment will be based on a cross sectional assessment of socio-economic status indicators: water, assets, maternal education and income index (WAMI). Infants will be declared to have EED (EED+ve) if they meet the criteria for both L:R and K:T ratios. Infants who do not meet the criteria for both L:R and K:T will be deemed to be EED free (EED –ve). ^{13}C SBT will be assessed in all infants at baseline. Additional data on weight, length/height, dietary intake, body composition and energy expenditure will contribute to understanding how EED impacts on nutrient intake and metabolism (reflected as body composition and energy expenditure) and the formulation of recommendations for energy and nutrient intake in the context of EED. Additional data on weight, length/height, dietary intake, body composition and energy expenditure will contribute to understanding how EED impacts on nutrient intake and metabolism (reflected as body composition and energy expenditure) and the formulation of recommendations for energy and nutrient intake in the context of EED.

Inclusion criteria for prospective cohort study: Infants (6-9 months of age) who are non-stunted (HAZ ≥ -1 to +2 SD) or at risk of stunting (HAZ < -1 to ≥ -2 SD) will be included in the cohort and screened for several indicators of socioeconomic status and EED. Only infants who are declared EED+ve or EED-ve by virtue of meeting both L:R and K:T ratio criteria will be followed up. Infants with intermediate results (ie those who meet the criteria for only one or the other ration will be excluded). These children will be followed monthly for three months to assess growth velocity. At three months children will be categorised into two groups: 1) children with EED (cases) and 2) children without EED (control)

based on a battery of validated tests.
At baseline a physician exam will be done to exclude active symptomatic illness (diarrhea, ALRI, symptomatic malaria, TB, etc). Children exhibiting stunting, HAZ <-2, and children with severe stunting, HAZ < -3, will be excluded. EED-ve infants will be excluded from the 12-month follow-up.

Sample size: We will include a convenient sample of 200 infants. Due the absence of data on EED and stunting, a difference of at least +0.2 in LAZ between EED+ve and EED-ve groups will be considered to be biologically relevant. The WASH Benefits Study in Kenya and Bangladesh assumed a +0.15 difference in LAZ[25]

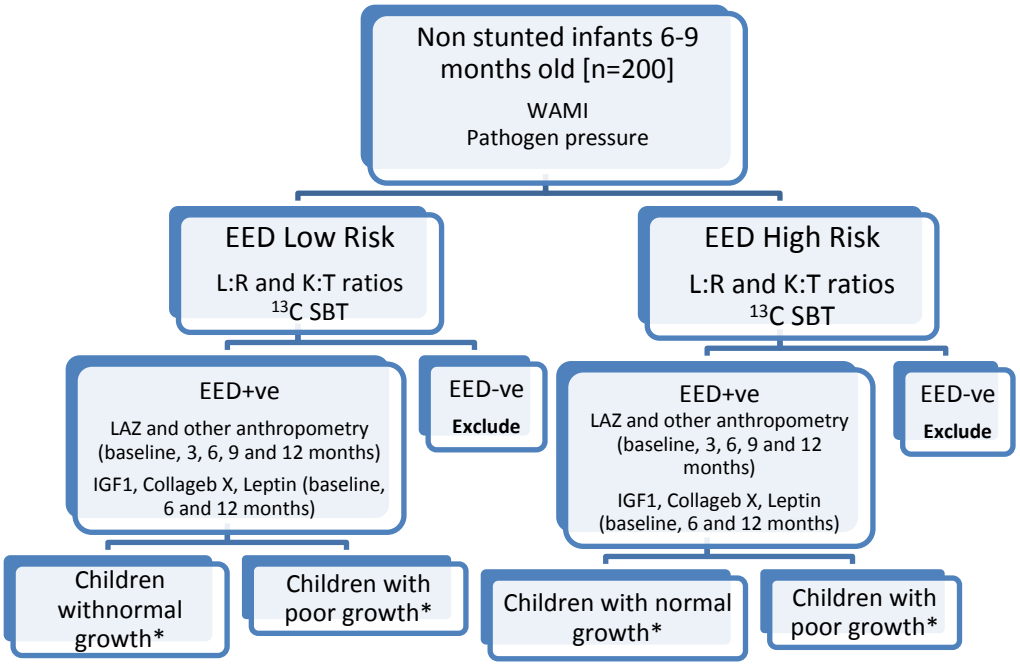

Figure 2: Proposed Flow Diagram for Phase 3

Analytical techniques

Despite the significance of EED to infant and child nutrition and health, biomarkers and simple diagnostic techniques for the definition and classification of EED are lacking. The gold standard for diagnosing EED, intestinal biopsy, is too invasive[19]. The most common, non-invasive test used to assess upper small bowel integrity is the dual sugar absorption test (lactulose:mannitol ratio), and has been employed in some intervention trials and functional studies. Attention is turning to the potential of applying stable isotopes in EED diagnosis, classification and management[20]. Using stable isotopes has significant potential in improving our understanding of EED and potentially could provide a non-invasive diagnostic test. Some practical challenges are still limiting the wide application of stable isotope techniques, such as costs and availability of stable isotopes tracers and cost, running and maintenance of instrumentation. However, newer spectroscopic technologies offer the potential point of care instrumentation solutions[21].

Stable isotopes have been used to assess gut dysfunction (small intestine bacteria overgrowth [SIBO], coeliac disease and chemotherapy induced small intestinal damage in rats) with different substrates (starch and other carbohydrates, mixed triglycerides, fatty acids, proteins, etc.). An example of a diagnostic ^{13}C breath test is the ^{13}C-urea breath test used to diagnose and monitor Helicobacter pylori infection in the stomach. The high specificity and sensitivity associated with the test makes it the ideal

non-invasive diagnostic technique[22]. The ^{13}C-sucrose breath test is a promising future technique to assess gut function and has been used in Australia to measure the absorptive capacity of the small intestine[23].

13C Sucrose breath test

^{13}C-sucrose breath test (SBT) has been applied as a simple, non-invasive test to measure intestinal absorptive function in humans and rodents based on the assay of total intestinal sucrose activity[24]. As eloquently summarised by Terry and co-workers, the release of ^{13}CO2 from ^{13}C-labeled substrates forms the basis for several noninvasive gut function tests, including those to assess gastric emptying, and gastrointestinal transit. In the SBT, the ^{13}CO2 in expired breath after oral ingestion of an appropriate substrate, in this case, sucrose which is metabolized to glucose and fructose by sucrase, is measured. After transit through the hepatic and respiratory systems, ^{13}CO2 is released. The expression of sucrase decreases in a proximal-to-distal gradient along the small intestine. As such, the SBT provides an integrated index of the total activity of sucrose throughout the small intestine. In a hospital-based prospective case-control study, the SBT was able to discriminate among Aboriginal children with diarrhea, asymptomatic Aboriginal children with an underlying environmental enteropathy, and healthy non-Aboriginal controls[23]. A practical case study of how this techniques works is the work by Abimosleh and others on rats. A baseline breath sample was collected at t = 0 following an overnight fast. Rats were then orally dosed with 1 mL of a 25% ^{13}C-labeled sucrose solution after which breath samples were collected every 15 minutes for 120 minutes. Breath samples were analyzed for ^{13}CO2 concentration using an isotope ratio mass spectrometer. Data were expressed as mean percentage cumulative dose of ^{13}C recovered at 90 minutes after sucrose administration25. The ^{13}C Sucrose Breath Test described above is based on the tracking of natural enrichment of sucrose with ^{13}C. It is postulated that higher ^{13}C enrichment would be required to have this technique applicable in EED settings. To do this, protocol optimisation and validation of the ^{13}C SBT against biopsy will be necessary. This CRP aims to improve our understanding of pathways underpinning EED and child growth through the application of a stable isotope based ^{13}C Sucrose Breath Test (SBT).

Dual sugar permeability test

The most common, non-invasive test used to assess upper small bowel integrity is the dual sugar absorption test (lactulose:mannitol ratio), or lactulose:rhamnose ratio, and has been employed in some intervention trials and functional studies. This test involves administration of an oral dose of both sugars (lactulose - 400mg/kg body weight and mannitol - 100mg/kg body weight)[13] followed by sequential urine sample collection for 5 hours. Lactulose, being a large disaccharide, is minimally absorbed via the paracellular route and then excreted unchanged in the urine. In conditions of altered intestinal permeability, lactulose traverses intercellular spaces, is cleared by glomerular filtration without reabsorption and can be measured in urine. On the other the sugar alcohol, mannitol, or monosaccharide, rhamnose, being smaller molecules, are passively and, at the doses used, usually completely absorbed in the small intestine[25]. However, in conditions of shortened intestinal villi, the absorption of mannitol is decreased and it is excreted in urine like lactulose[26]. Urinary concentrations of lactulose and mannitol can be measured using gas-liquid chromatography. Altered intestinal permeability is defined as a ratio of urinary recovery of lactulose to mannitol [L:M] > or =0.0727. L:M ratio is susceptible to season, geographical and intra-patient variation[25]. L:M ration should be augmented with other biomarkers to enable population screening, individual case diagnosis and monitoring of the consequences of EED[26]

Kynurenine/Tryptophan (K:T) ratio

Ninety five percent of dietary tryptophan is metabolized in various cells (including somatic tissues such as the lungs and intestines and cells of the immune system) via the kynurenine pathway, a major link between the immune and nervous systems.[28] The degradation of tryptophan to kynurenine is catalysed by indoleamine 2,3 dioxygenase-1 (IDO1)29. IDO1 expression is induced by inflammatory stimuli such the cytokines TNF-α or IFN-γ[26]. Several gastrointestinal diseases including the inflammatory bowel diseases (IBDs) and malignancy are associated with upregulation of IDO130. Inflammatory disease state is associated with a modest reduction in serum tryptophan, increase in serum kynurenine, or a combination of the two29. As such the application of serum Kyn-to-Trp ratio (K:T ratio) as a biomarker of EED is being explored. Indeed, emerging evidence from the MAL-ED study shows that tryptophan is low in children Tanzania and Peru; stunted children in these populations have lower levels of all essential 9 amino acids than non-stunted children. Kyn/Trp ratio. is associated with oral polio virus 1 (OPV1) vaccine failure. Increase of 1 SD in kynurenine associated with 1.90 times higher risk of OPV1 failure (95%CI 1.18-3.10) in a model that controlled for length-for-age z score (LAZ) at time of biomarker measure, breastfeeding, number of days of diarrhea up to date of primary vaccine administration, and diarrhea at the time of primary immunization. The concentrations of Trp and Kyn can be measured simultaneously by liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). The IDO activity was estimated by calculating the serum Kyn-to-Trp ratio (Kyn/Trp ratio)[31].

Assessment of environmental risk factors for EED

The WAMI Index

Reported environmental risk factors for EED include poor socioeconomic status, an unprotected source of drinking water, use of facilities for disposal of feces that sequester it from the environment, close, prolonged, frequent contact with domesticated animals, food insecurity, poor dietary quality. Additional risk factor for EED are a weight-for-height z score (WHZ) < 0, but >-2.

A validated questionnaire such as the water, assets, maternal education and income index (WAMI)32 is preferred. When measured across eight sites, a 25% difference in WAMI was associated with a change in length-for age z score (HAZ) of 0.3 (95%91 0.22, 0.55) cross-sectionally. Advantages of this strategy include ease of application, validation in a multisite study, and quantified relationships between two biomarkers of EE, the lactulose:mannitol z score (L:M-Z) and the number of pathogens in a non-diarrheal stool collected at the time of the LM test. The WAMI score was inversely associated with L:M-Z, with a 1 unit increase in WAMI (from lowest to highest) associated with a 0.43 lower L:M-Z. There was a statistically significant interaction between WAMI and age, with the greatest effect of WAMI on L:M-Z occurring among the older (9 and 15 month old) children[32].

Pathogen pressure

The number of pathogens in stool is directly correlated with the lactulose mannitol z score (LMZ). It is essential for competitive proposals to characterize their high risk and low risk population in detail in relation to baseline anthropometric status, socioeconomic status, intensity of enteric infections, and water and sanitation assessments in order to ensure that these groups represent different phenotypes. Pathogen presence, a Taqman Array Cards to the Diagnosis of Respiratory Diseases (TACCARD) diagnostics on 3 stools collected 1 week apart[33]. Children at high risk for EED will be compared to children at low risk for EED in one or more of the domains utilizing stable-isotope technology. High risk 6-18 month old children will be identified by demographic and behavioral risk factors listed above or on the basis of local data from the location form which the study is

proposed. For example, a risk child might have a WAMI index in the bottom quartile (0.25 or below).

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Overall Objective

To improve our understanding of the pathways underpinning the relationship between EED and child growth for improved health

Specific Research Objectives
To obtain new information on the diagnosis and classification of EED using a stable isotope breath test and on how EED affects growth in infants and young children

Outcomes
Better understanding of the pathways underpinning the relationship between EED and child growth to enable design of interventions for prevention and treatment of EED for improved health

Outputs
1. Non-invasive 13C Sucrose Breath Test to diagnose EED and assess its effects on health
2. New data on the pathways underpinning the relationship between EED and child growth including dietary, mucosal integrity/permeability and nutrient and energy partitioning.
3. Publications in the form of scientific reports and peer-reviewed papers and conference presentations.
4. Preparation of an IAEA Human Health Series publication on 13C Sucrose Breath test to diagnose EED.

Assumptions
1. Partnerships (North-South and South-South) established between local institutes implementing the CRP and EED and nutrition researchers,
2. Adequate budget available for the entire period of the CRP

Proposal submission forms
Research institutions in Member States interested in participating in this CRP are invited to submit proposals directly to the Research Contracts Administration Section (NACA) of the International Atomic Energy Agency: Official.Mail@iaea.org or to Mr Victor Owino: V.Owino@iaea.org
The forms can be downloaded from http://cra.iaea.org/cra/forms.html. For more information about research contracts and research agreements, please visit our web-site

Deadline for Deadline for submission of proposal
Proposals must be received **no later than 30 April 2017**. Transmission via Email is acceptable if all required signatures are scanned.

For additional information, please contact:
Mr Victor Owino, Nutrition Scientist
Nutritional and Health-related Environmental Studies Section Division of Human Health International Atomic Energy Agency (IAEA) A-1400 Vienna, Austria Phone: + 43 1 2600 21657 Fax: + 43 1 2600-7
V.Owino@iaea.org